Venous Control In A Primitive Fish

Eptatretus cirrhatus

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by

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0.1 Abstract

Only a small amount of the available literature is concerned with venous control in lower vertebrates, such as fish. It has even been suggested that veins in fish are not important factors in active regulation of venous return.

Preliminary work carried out for this thesis strongly refuted this assumption, highlighting gaps in the existing literature. As a result of the lack of information pertaining to the physiology of the central venous compartment of the circulation, my objective has been to investigate various aspects of this in the hagfish *Eptatretus cirrhatus*.

Hagfishes, with the lowest arterial blood pressures and highest blood volumes amongst the chordates, are the earliest surviving group to separate off from the chordate lineage. They provide a unique opportunity to investigate likely physiological mechanisms in ancestral chordates. The data presented in this thesis suggest that 1) *E. cirrhatus* exhibit some cardiovascular compensation during volume manipulation, however this only occurs with volume loading and not during volume depletion, 2) Veins from *E. cirrhatus* can respond vasoactively to adrenergic stimulation *in vitro* and 3) Plasma catecholamines in *E. cirrhatus* also respond to volume manipulation and provide a potential *in vivo* mechanism for the control of changes in cardiovascular parameters that were observed during volume loading.

Chapter 1

Why Hagfish are Special

1.1 Why Hagfish Are Special

1.1.1 Hagfish are Osmoconformers

Many studies have reported that hagfish, in contrast to teleost fishes are unable to regulate their osmolarity independent of their environment by controlling Na^+ and Cl^- concentrations. They are generally considered to be osmoconformers and the work presented in this thesis supports this finding (McFarland and Munz 1958; Cholette and Gagnon 1973; Robertson 1974).

Even though these animals are traditionally thought to be osmoconformers, it has been repeatedly reported that the Na^+ concentration of serum is higher than that of the external environment (by 10 to 19%) (Robertson 1974), a situation similar to that of vertebrates in fresh water. This is interesting as hagfish kidneys produce an almost isotonic urine and cannot reabsorb significant levels of Na^+ via the kidney. It is thought that specific "Mitochondria Rich" cells in the epithelium of the gill, may be responsible for the uptake of Na^+ from the marine environment (Elger 1987,Mallatt and Paulsen 1986). The definite function of these cells has as yet not been ascribed, however, these are the only cells with the pronounced ultrastructural characteristics of ion transporting cells. It has been postulated that the higher Na^+ concentration of hagfish serum may arise from acidotic conditions and the need of these animals to excrete excess H^+ via an antiporter.

1.1.2 Pressure, Flow and Blood Volume

Hagfish have a circulation that has been characterized as a low pressure moderate flow system, despite possessing large blood volumes and multiple hearts (Forster et al. 1991). Arterial pressures measured in hagfish are the lowest recorded in the phylum vertebrata (Table 1), and the work presented in this thesis supports this finding. There is not much information about venous pressures in hagfish and the work presented here suggests that venous pressures are also low.

The hagfish heart generates low forces and is particularly sensitive to increases in afterload (Johnsson and Axelsson 1996; Johnsson et al. 1996). However, a low pressure moderate flow circulatory system seems to preclude a requirement for a heart that can generate much force and has energy conserving advantages. As mentioned, hagfish also have the largest blood volumes in the phylum vertebrata with reported values of 187ml/kg and 177ml/kg (McCarthy and Conte 1966; Forster et al. 1989; Forster et al. 2001). Low venous PO_2 values supports a relatively long turn over time for the circulation in that blood may spend long periods in the tissues (Wells et al. 1986).

1.1.3 Hypoxia Tolerance and Low Metabolic Rate

It has been assumed that hagfish are physiologically and biochemically adapted to withstand hypoxia and that this group retains a well developed anaerobic capacity (Hansen and Sidell 1983; Forster 1990). However there may be some differences between species

in their ability to tolerate hypoxia (Forster 1998). Specifically it is thought *E. cirrhatus* may not be as well adapted to cope with this challenge as is *Myxine glutinosa* which is known to frequent hypoxic environments. Work presented in Chapter Four supports this hypothesis as extremely high plasma catecholamines were associated with hypoxia in *E. cirrhatus*, greater than had previously been measured in *M. glutinosa* undergoing hypoxic challenge (Perry et al. 1993).

The Multi-Compartmented Venous System

Hagfish have a series of venous sinuses, one of which (the subcutaneous system) contains a large proportion of the total blood volume (Forster 1997). The physiological implications of this sinus with regard to cardiovascular parameters during volume manipulation is discussed in Chapter Four. Many likely physiological roles for this compartment have been suggested but there is still a lack of information on how this system might operate in terms of cardiovascular control and its relation to the central circulation.

Blood Pressure Comparisons			
Species	**Condition**	**Pressure cm H_2O**	**Reference**
Myxine glutinosa (hagfish)	Resting	DA = 7.85	Axelsson et al. (1990)
		VA = 10.61	
Eptatretus cirrhatus (hagfish)	Resting	DA= 10.91	Forster et al. (1998)
		VA= 14.68	
	Swimming	DA= 10.20	
		VA= 15.81	
Eptatretus cirrhatus	Normoxia	DA= 13.26	Forster et al. (1992)
		VA= 16.32	
	Hypoxia	DA= 14.28	
		VA= 22.43	
Entosphenus tridentata (lamprey)	-	DA= 25.49-43.85	Johansen et al. (1973)
Scyliorhinus canicula (dogfish)	Resting	DA= 39.77	Short et al. (1979)
		VA= 52.01	
	Hypoxia	DA= 32.63	
		VA= 43.85	
Gadus morhua (Atlantic cod)	Resting	DA= 32.63	Axelsson and Nilsson (1986)
		VA= 49.97	
	Swimming	DA= 40.79	
		VA= 63.22	

Table 1.1: Blood pressures in various fishes. Based on Table 15.1 in Forster 1998 Cardiovascular function in hagfishes: In The Biology of Hagfishes. All pressures have been converted from kilopascals (kPa) to centimeters of water (cm H2O) for the purposes of comparison.

1.2 The Anatomy of the Venous System

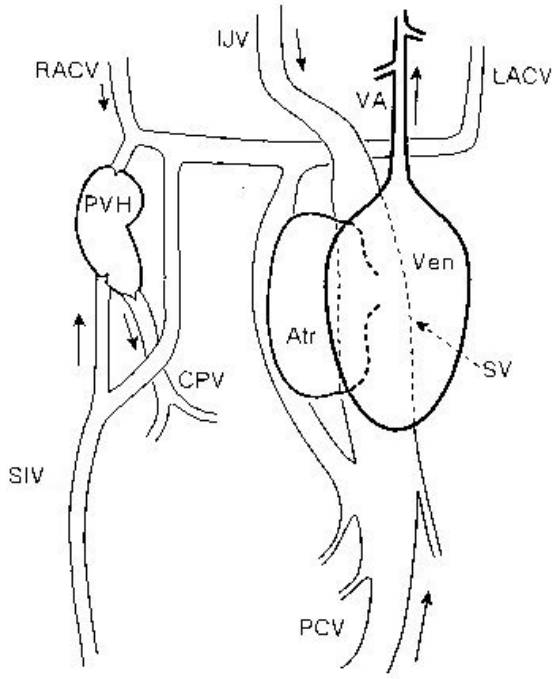


Figure 1.1: Representative diagram showing the arrangement of the systemic heart, the portal heart and associated venous vasculature of *Myxine glutinosa.*
Atr, atrium; CPV, common portal vein; IJV, inferior jugular vein; LACV, left anterior cardinal vein; PCV, posterior cardinal vein; PVH, portal vein heart; RACV, right anterior cardinal vein; SIV, supraintestinal vein; SV, sinus venosus; VA, ventral aorta; Ven, ventricle. Reproduced from Forster et al. 1991

The corrosion casts presented in this chapter revealed a similar vascular arrangement in *E. cirrhatus* to that proposed for *M. glutinosa*(see Figure 1.1), with a few key differences. Note that the PCV exists as two parallel branches with a number of interconnecting anastomoses running the length of the parallel vessels in *E. cirrhatus* (see Figure 1.3). The two branches of the PCV collect venous blood from the posterior of the animal and pass it forward to the sinus venosus of the systemic heart. Hence blood pressure in the PCV is likley to influence filling of the systemic heart, especially as *E. cirrhatus* appears to rely on *vis-a-tergo* forces to fill the heart(see discussion Chapter Two). The systemic heart also receives inputs from the left ACV (see Figure 1.4, white repeating arrows) and the venous effluent from the liver. The cast in Figure 1.4 was filled through both ACVs, the left ACV was filled with red casting material and the right ACV was filled with blue. Although some mixing has occurred (as the resin takes ¿10 minutes to cure), the systemic heart appears proportionally more red as it links directly (via the sinus venosus) to the left ACV. Conversley the portal heart (PVH) and SIV has filled (in a retrograde fashion) mostly with blue casting material, demonstrating a link to the right ACV. In *E. cirrhatus* the ventral aorta divides as it exits the ventricle whereas in *M. glutinosa* it is a single vessel leaving the ventricle. From the ventral aortas afferent branchial arteries

carry deoxygenated blood to the gill pouches (see Figure 1.6, left and right ventral aortas and afferent branchial arteries). Figure 1.6 demonstrates the branching pattern of the vasculature supplying the gill pouch. ABAs bifurcate into two main arterioles from which finer afferent arterioles and cappillaries radiate. Blood can exit the gill pouch by two routes 1) the arterio-arterial route via the efferent branchial arteries and on into the dorsal aorta or 2) the arterio-venous route which allows blood to move into the venous sinus system. The portal heart receives blood from the SIV and as previously mentioned the right ACV (see Figure 1.4, black repeating arrows). It pumps to the liver via the common portal vein (see Figure 1.5) and is thought to provide kinetic energy to aid the perfusion of the liver. However, Davison (1995) reports finding an apparently healthy specimen of *E. cirrhatus* without a portal heart suggesting this pump may not be an essential requirement for life.

These casts revealed another key difference between the vascular anatomy of *M. glutinosa* and *E. cirrhatus*. The casts presented in this chapter do not identify an anastamosis between the left and right ACV as seen in *M. glutinosa* (see Figures 1.1 and 1.4). The functional significance of blood flow off the gut and into the the SIV and on into the liver is clear and the arrangement favors uptake of the products of digestion and absorption at the liver. What is not so clear is the significance of the pathway that venous blood takes from the RACV via the portal heart and liver to be returned to the systemic heart. Does the presence of an anastomosis between the right and left ACV in *M. glutinosa* allow for faster cardiac filling by allowing the return of blood from the anterior periphery to avoid the presumably high resistance of the capillary beds of the liver? Clearly the functional significance of these different vascular routes would benefit from further study.

The figures below are corrosion casts made of the venous anatomy of *E. cirrhatus*. They illustrate the hearts and parts of the vasculature most commonly discussed in this thesis.

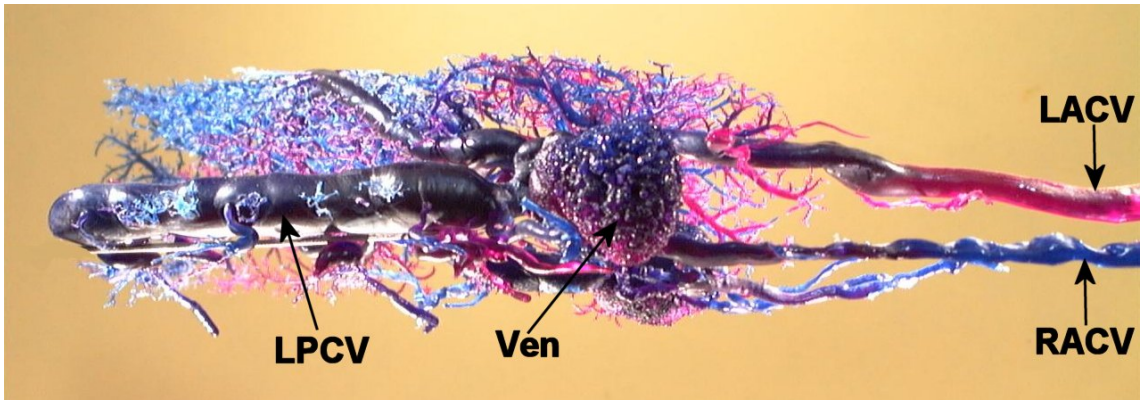


Figure 1.2: Corrosion cast showing arrangement of parts of venous system of *E. cirrhatus*. **Dorsal view**, the animals' anterior is to the right. LACV= left anterior cardinal vein (red), RACV= right anterior cardinal vein (blue), LPCV= left branch of the posterior cardinal vein and Ven= ventricle of the systemic heart (note traburculated structure).

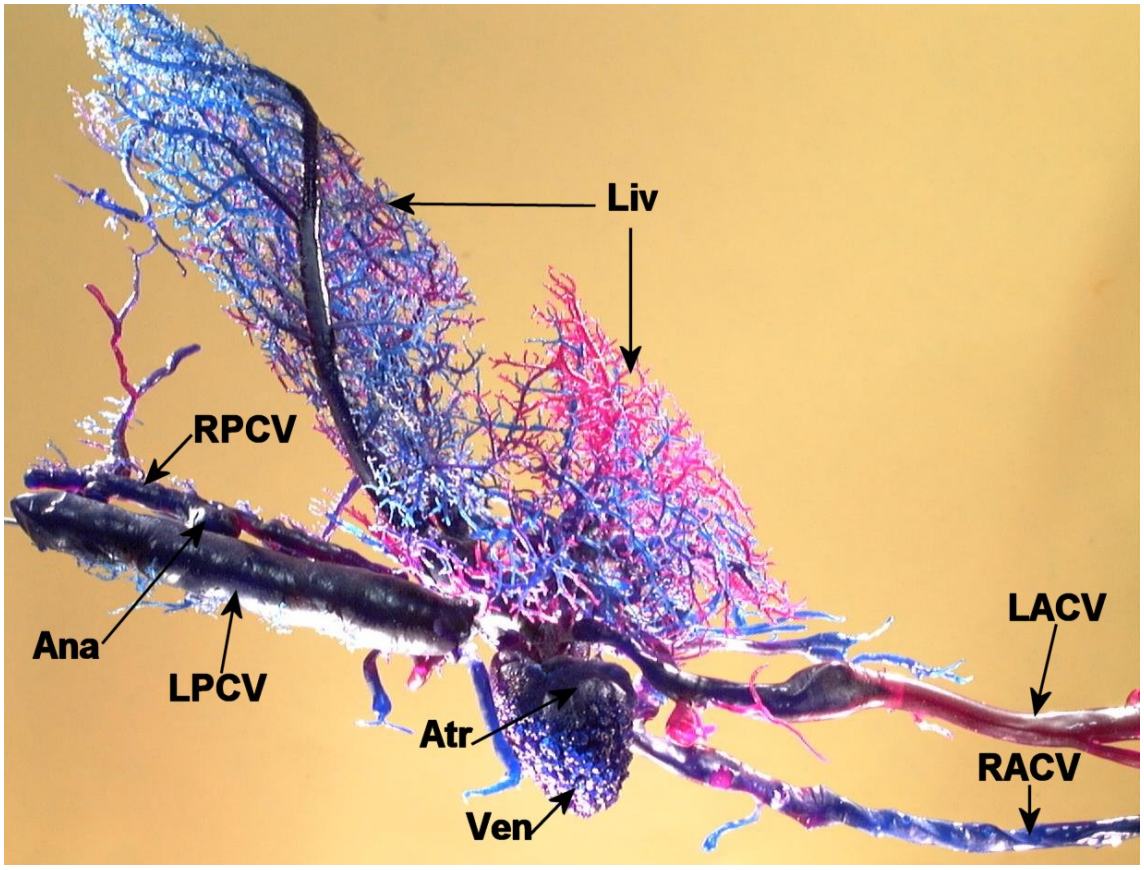


Figure 1.3: Corrosion cast showing arrangement of parts of venous system. **Ventral-lateral view** looking onto the left side of the circulation with the animals' anterior to the right. LACV= left anterior cardinal vein (red), RACV= right anterior cardinal vein (blue), LPCV= larger left branch of the posterior cardinal vein, RPCV= smaller right branch of the posterior cardinal vein, Ana= anastamosis between the LPCV and RPCV, Ven= ventricle of the systemic heart, Atr= atrium of the systemic heart and Liv= vascular beds of the liver (note blue stained vasculature in the liver represents the afferent supply from the portal heart and SIV, and the red stained vasculature represents the efferent supply draining to the sinus venosus of the systemic heart).

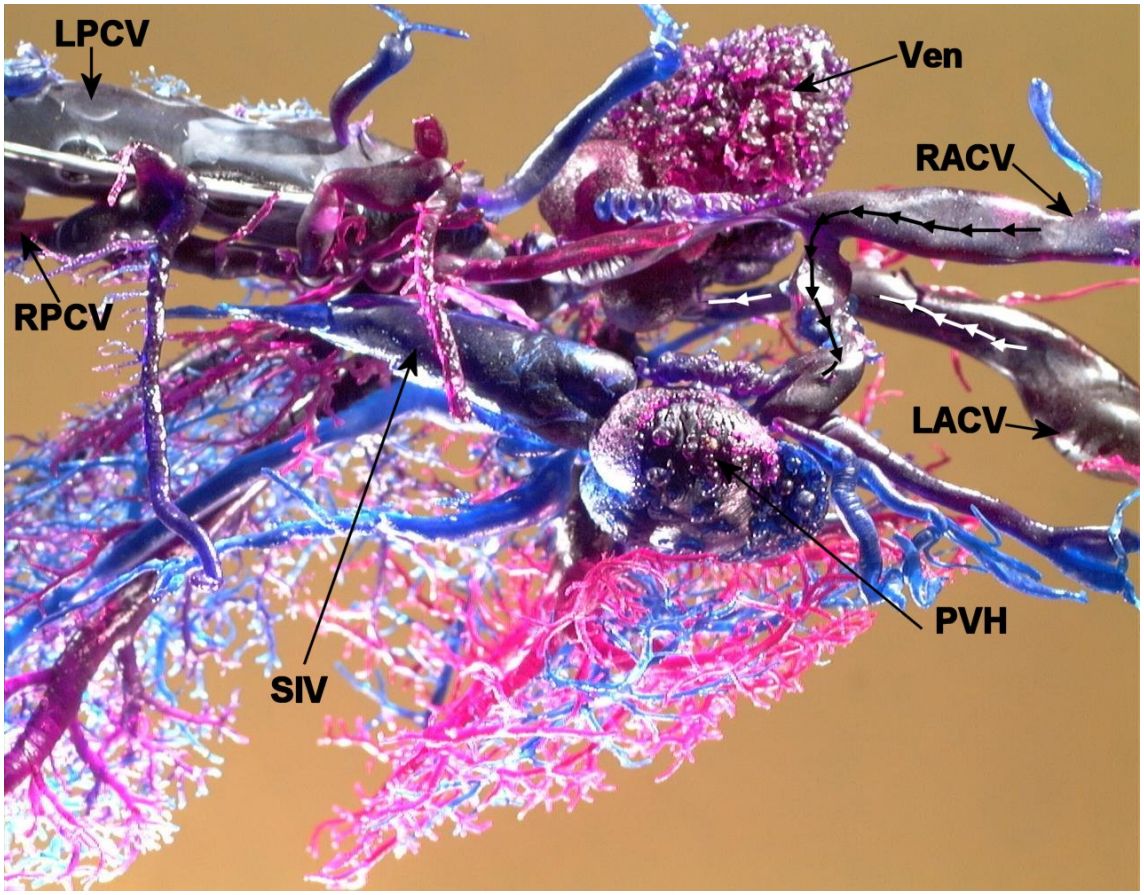


Figure 1.4: Corrosion cast showing arrangement of parts of venous system. **Ventral-lateral view** looking onto the right side of the circulation with the animals anterior to the right. LACV= left anterior cardinal vein, RACV= right anterior cardinal vein, LPCV= larger left branch of the posterior cardinal vein, RPCV= smaller right branch of the posterior cardinal vein, Ven= ventricle of the systemic heart, PVH= portal vein heart and SIV=supraintestinal vein. White repeating arrows show the LACV supplying the systemic heart, whilst black repeating arrows show the RACV supplying the portal heart, (note, there is no anastamosis between these two vessels).

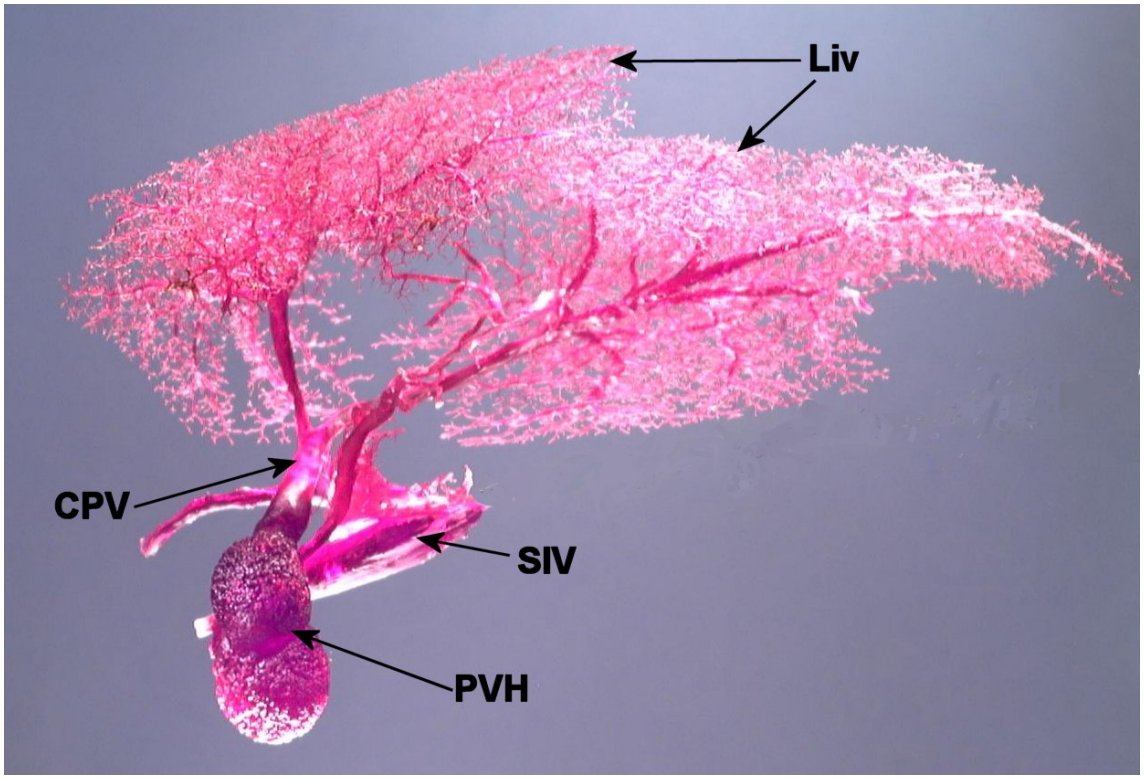


Figure 1.5: Corrosion cast showing arrangement of parts of venous system. **Lateral view** looking on to the left side of the circulation with the animals' anterior to the left. PVH= portal vein heart, SIV= supraintestinal vein, CPV= common portal vein (which carries the afferent blood supply to the liver) and Liv= vascular beds of the liver.

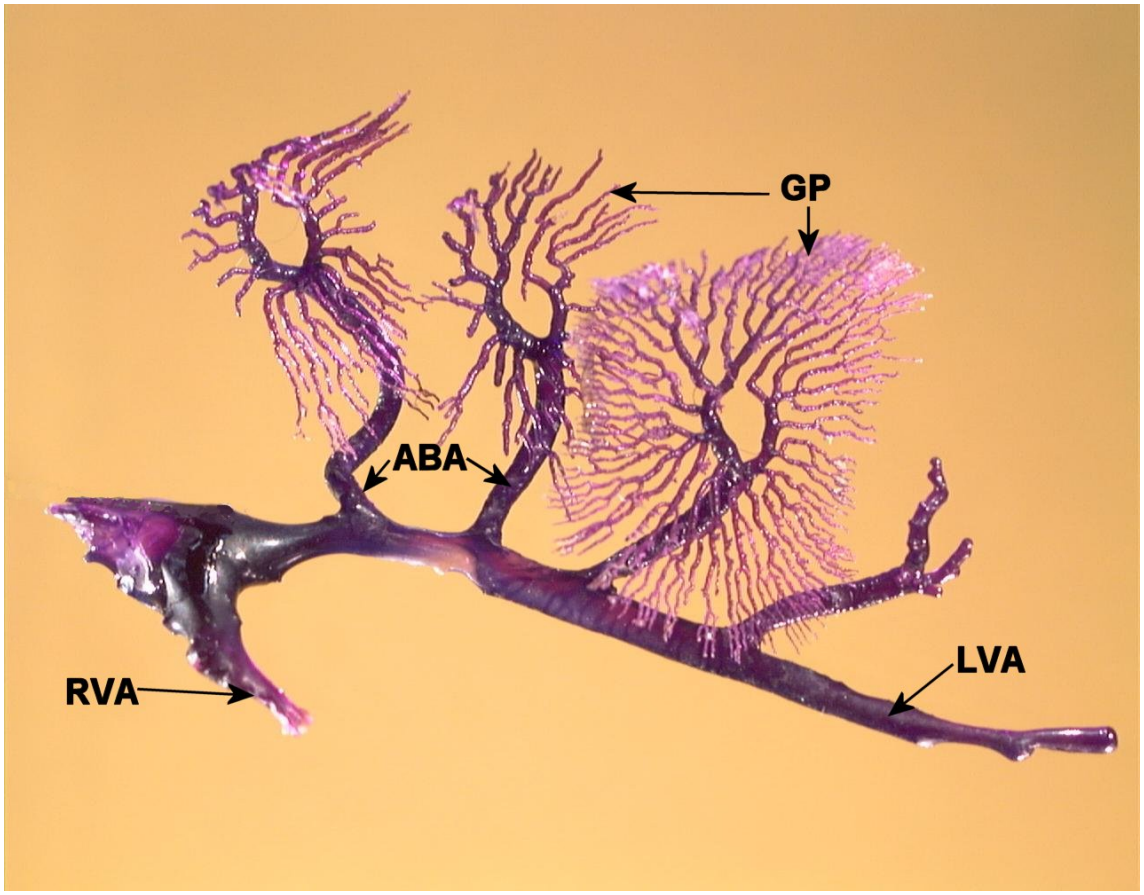


Figure 1.6: Corrosion cast showing arrangement of parts of the branchial vasculature. **Lateral view**, animals' anterior to the right. RVA= incompletely casted right ventral aorta, LVA= left ventral aorta, ABA= afferent branchial arteries and GP= gill pouch vasculature.

Chapter 2

In Vivo Volume Manipulation

2.1 Introduction

The cardiovascular system of the hagfish has major differences from those of other vertebrates and because of this attention has been drawn to certain aspects of cardiovascular physiology in this animal. Cardiac function, metabolic rate, hypoxia tolerance and the venous sinus system have all been investigated to varying degrees (Satchell 1986; Davie et al. 1987; Forster et al. 1989; Axelsson et al. 1990; Forster 1990; Forster et al. 1992; Johnsson and Axelsson 1996; Forster 1998).

Although a number of studies have measured *in vivo* arterial pressures in hagfish, there is a general lack of information about events in the central venous compartment of these animals (Axelsson et al. 1990; Forster et al. 1992; Perry et al. 1993). Hagfish hearts exhibit a Frank-Starling mechanism and *in situ* studies have shown that changes in preload markedly affect cardiac output, so emphasizing the importance of central venous pressure (Johnsson and Axelsson 1996; Johnsson et al. 1996).

In view of the lack of information regarding this aspect of hagfish physiology, experiments were carried out to assess pressure in the central venous compartment (PCV) of *E cirrhatus* at rest and during volume manipulation experiments. Pressures were also simultaneously measured in the dorsal aorta and supraintestinal vein so that changes in the central venous compartment could be correlated with events in other parts of the circulation.

Measurement of resting pressures confirmed that *E cirrhatus* possesses extraordinarily low pressures in all parts of the circulatory system that were investigated. Volume manipulation experiments revealed the unexpected and intriguing result that central venous pressures were reduced during volume loading and that this could be correlated with the return to resting values in other parts of the circulation later in this treatment. During volume depletion no compensatory increase in central venous pressure was observed and in general vascular pressures were depressed for the duration of this treatment.

2.2 Methods

2.2.1 Experimental animals

The New Zealand hagfish, *Eptatretus cirrhatus* Forster, was used in all experiments. Hagfishes were collected off Motunau beach or Akaroa harbour, New Zealand and were transferred to the University of Canterbury in Christchurch where they were held in aquaria containing circulating sea water. They were held at least one week prior to experimentation, and were not fed during this period. In total 25 animals with an average weight of 1073±63g (±S.E.M.) were used for this work. Animals were kept at 12-14 $^{\circ}$C under a 12 hour light: dark cycle.

2.2.2 *In Vivo* Pressure Measurement During Volume Manipulation Experiments

Blood pressures were measured in hagfish at rest and during volume manipulation to assess what affect these treatments had on cardiovascular parameters.

Surgical Method

Hagfish were anaesthetized in seawater containing MS222 (ethyl-*p*-aminobenzoate and 3-aminobenzoic acid ethyl ester metanesulfonate 0.4g L^{-1}) and benzocaine (0.4g L^{-1}). After approximately 60min, the fish were transferred to an operating table where a small mid ventral incision was made to expose and allow cannulation of the dorsal aorta (DA), posterior cardinal vein (PCV) and supraintestinal vein (SIV).

Vessels were cannulated non-occlusively with Portex polythene tubing (0.86mm ID, 1.27mm OD) which were reversibly connected to disposable pressure transducers via a length of larger diameter polythene tubing (1.0mm ID, 2.0 OD). The open ends of cannulae were advanced towards the systemic heart in the DA and PCV, and towards the portal heart for SIV cannulation. Cannulae were threaded through a small cuff of latex rubber, which was secured to surrounding tissue with superglue to hold cannulae in place. Cannulae contained heparinised, hagfish HEPES buffered saline (HHBS) with the following composition in g.l $^{-1}$: 27.70 NaCl, 0.60 KCl, 0.75 $CaCl_2$.2 H_2O, 0.75 $MgSO_4$.7H_2O, 0.72 HEPES acid form, 1.82 HEPES sodium salt, 1.00 glucose, pH 7.8).

After surgery animals were placed in individual holding tanks and a steady flow of aerated seawater was passed across the gills by means of a tube inserted into the nasal opening until the animals regained consciousness.

Volume Manipulation

After a 24 hour recovery period, simultaneous recordings of resting blood pressure were taken from all three vessels prior to and after the induction of volume manipulation. Blood pressure and heart rates were measured using PVB Medizintechnix Kirchseeon DPT-6003 disposable pressure transducers (Jackson and Allison Auckland). Signals were suitably

amplified and recorded with Power Lab software and hardware. All transducers were maintained at ambient water temperatures and were calibrated against a static water column, where zero pressure was set to the level of the seawater in the tank.

Volume manipulation was achieved by turning off the recirculating seawater supply to the tank, followed by the addition of a measured quantity of either distilled water or dissolved sea salt to change the salinity of the tank water from 100%, to approximately 90% or 110% seawater. This treatment was not expected to directly affect intravascular pressures by producing an exact 10 percent change in this parameter as a number of factors may influence this (i.e. stressed vs unstressed volume, interstitial fluid load, tone and compliance etc). The tank water was continuously aerated with an air stone, once the recirculating seawater supply had been switched off. Tanks contained between 30L and 40L of bathing medium.

The tank water was slowly changed to the experimental medium ($\sim$110% or $\sim$90% seawater) 1-2 minutes before time 0 to allow a gradual introduction of the new medium over 2 minutes for all volume manipulation experiments.

Simultaneous pressure recordings of all three vessels was carried out for a minimum of 150 minutes after volume manipulation. In some cases animals were exposed to a prolonged volume manipulation so pressures could be measured at 24 hours.

2.2.3 Statistical Analyses

Statistical analyses used are stated in figure legends. To generate figures, pressures recorded in each vessel were averaged over 10 minute periods.

All statistical tests were two tailed unless otherwise stated and all means are presented $\pm$S.E.M. The limit of significance was $P<0.05$ unless otherwise stated.

2.3 Results

2.3.1 Changes in Plasma Osmolarity with Volume Manipulation

Average Percentage Change of Plasma Osmolarity With ~10% Change of external Medium Osmolarity

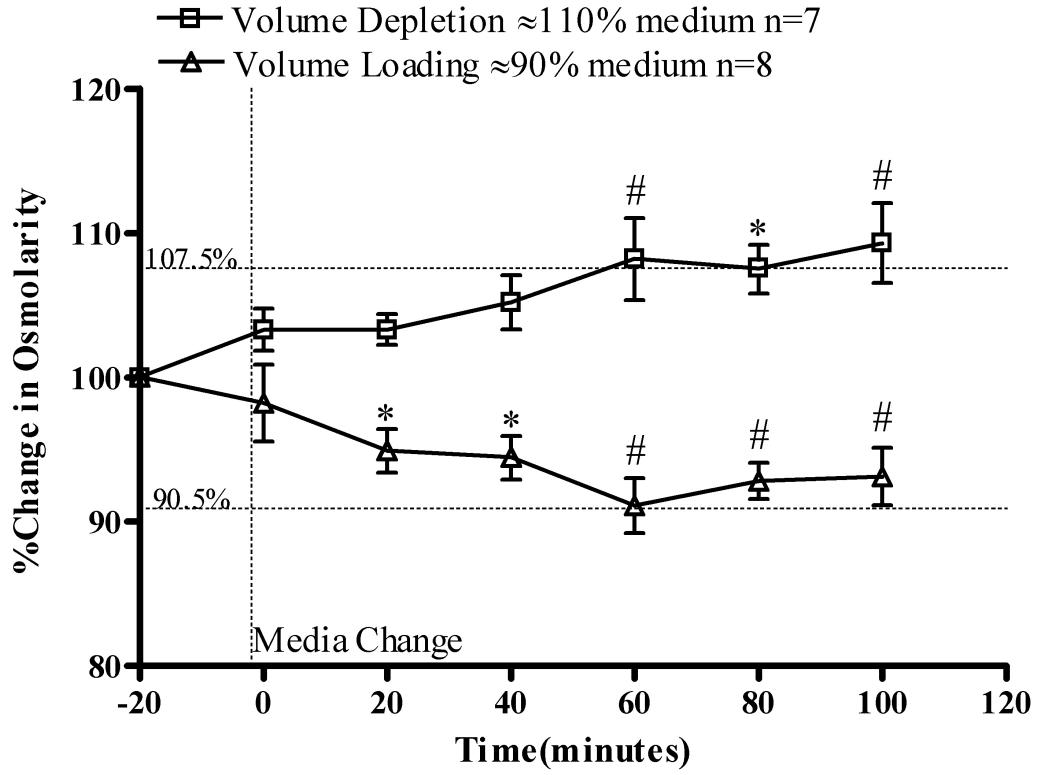


Figure 2.1: Change in hagfish plasma osmolarity with a ~10% change in external media osmolarity. * indicates significant difference to pre-change values (Dunnett's Multiple Comparisons post test #=P<0.01, *=P<0.05).

The experimental medium (tank water) was changed (~110% or ~90% seawater) 1-2 minutes before time 0 (dashed vertical line) to allow a gradual introduction of new media over 2 minutes.

The osmolarity of the external medium was measured before and after alteration to allow confirmation that a change of approximately 10% had occurred. The average changes in osmolarity of the external medium for figure 2.1 are indicated by dashed horizontal lines with values associated. Plasma osmolarity was measured at 20 minute intervals, starting 20 minutes prior to and finishing 100 minutes after, external medium change.

The data for figure 2.1 were not collected from the same animals used to create the rest of the figures in this chapter, as the focus there was to measure blood pressures and the collection of blood samples may have interfered with this. Results of serial blood sampling experiments to measure plasma catecholamines during volume manipulation, matching

data from figure 2.1 can be viewed in Chapter Four.

Although plasma samples were not taken, osmolarity of tank water was measured before and after experiments in this chapter. Upon volume depletion there was an average increase in tank water osmolarity to 111.2% of pre-change osmolarity (S.E.M=0.65 n=11), with very little change by 24hrs to 111.1% (S.E.M.=0.68 n=11). For volume loading, there was an average decrease in tank water osmolarity to 87.97% of pre-change osmolarity (S.E.M.=1.16 n=10), also with very little change by 24hrs to 87.52% (S.E.M.=1.26 n=10).

During our experimental manipulations, external osmolarity was not always changed by exactly 10%. However, our measurements have produced values that do not deviate appreciably from this target change. We therefore accept a change of $\sim$10% has occurred and for convenience refer to volume depletion as an osmotic challenge of 110% of resting values and volume loading as 90%.

Repeated Measures Analysis of Variance (ANOVA) was performed for data in figure 2.1. Changes in plasma osmolarity were significant on volume loading and depletion, with P values of 0.0001 and 0.0026 respectively. Both data sets were also significant when tested for linear trend ($P<0.0001$) with slope values of -1.258 (loading) and 1.468 (depletion).

It can be seen from figure 2.1 that hagfish plasma osmolarities change rapidly with changes in the external media. Plasma osmolarity matches that of the external media and becomes most stable by 60 minutes, all plasma osmolarities are significantly different to prechange values by 60 minutes. Plasma osmolarities become significantly different from prechange values by 20 minutes on volume loading, however significance occurs at 60min for volume depletion.

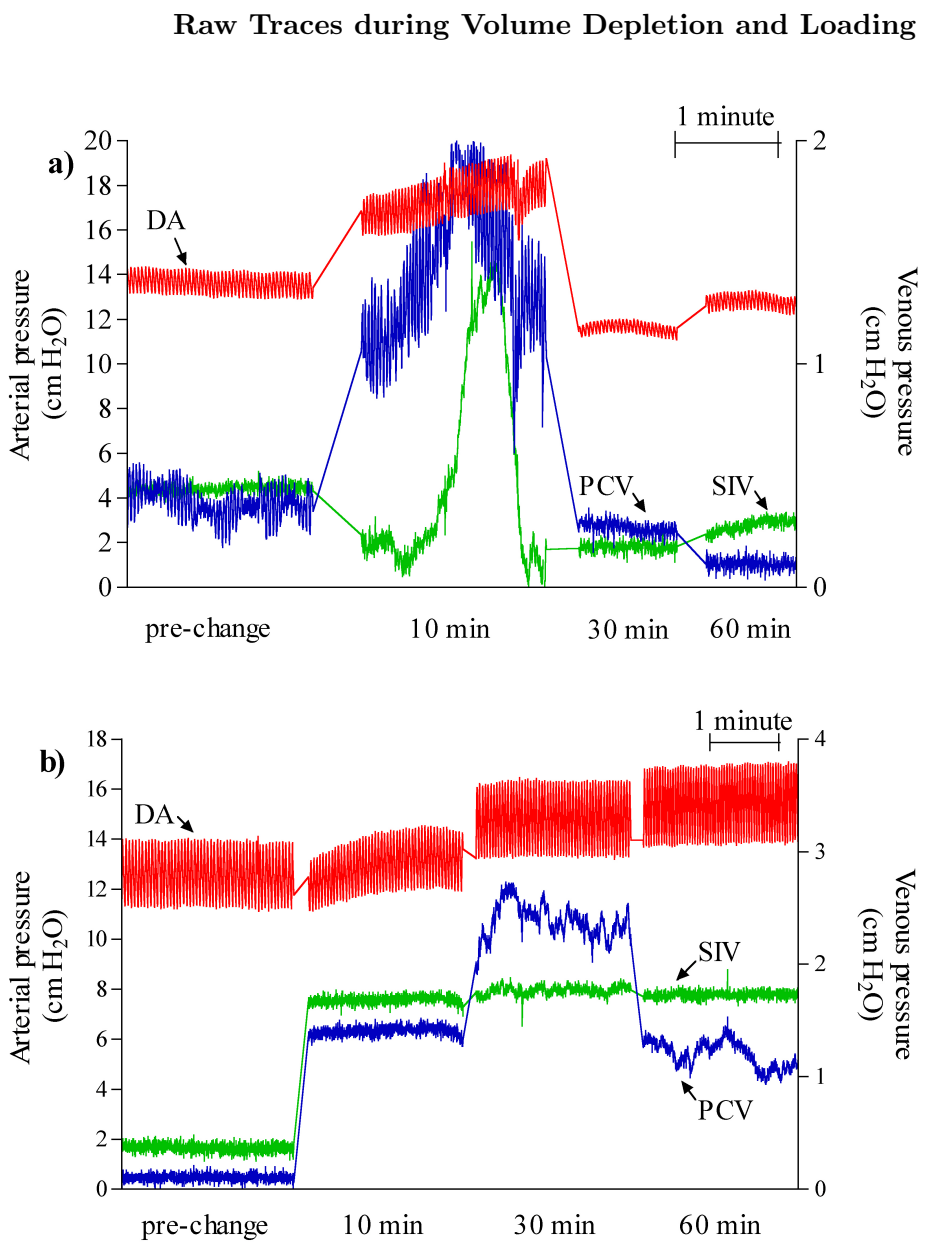


Figure 2.2: Representative raw traces from the same fish showing blood pressure responses of three vessels to, **a)** volume depletion and **b)** volume loading. Arterial pressures are displayed on the left axis, and venous pressures on the right. DA=Dorsal Aorta, PCV=Posterior Cardinal Vein, SIV=Supra-Intestinal Vein.

In figure 2.2 both graphs display typical prechange/resting pressures for the three vessels displayed. Average resting DA pressure from the depletion graph was 13.63cm H_2O, close to the mean DA pressure in 17 hagfish of 14.09 ±0.46cm H_2O (Table 2.1). The PCV resting values of 0.38 and 0.1cm H_2O and SIV values of 0.44 and 0.37cm water (for depletion and loading respectively) are also comparable with mean values for PCV of 0.39 ±0.08cm H_2O taken from 15 fish and SIV 0.67 ±0.14cm H_2O taken from 13 fish (Table 2.1). Proportional changes in venous pressures were greater than for arterial pressures but not in absolute terms.

Vascular Pressures During Volume Depletion in One Fish

Figure 2.2a displays some of the common artifacts seen upon volume depletion. It can be seen that at 10 minutes pressure in all three vessels substantially increased rather than decreasing as might be expected (17.52, 1.43 and 0.51cm H_2O for DA, PCV and SIV respectively). This phenomenon occured simultaneously with increased activity levels which seemed to be a reaction to the change in medium in some animals. Animals may be seen to exhibit swimming and burrowing behaviours at this time especially on volume depletion (more so than on loading). This physical activity has the effect of substantially raising central venous pressure, proportionally far more than for central arterial or SIV pressure. Generally this activity was transient if it did occur and did not have a lasting effect on the cardiovascular system. Following the bout of activity at 10 minutes it can be seen from Figure 2.2a that at 30min and 60 min pressures in all three vessels have dropped below initial prechange values. At 60 min pressures are 12.79, 0.1 and 0.28 cm of water for DA, PCV and SIV.

Vascular Pressures During Volume Loading in One Fish

There is a progressive increase in DA pressure until 60 minutes in this animal. Pressure reached an average value of 15.39cm H_2O, 122% of the prechange value. Venous pressures can also be seen to increase, with values at 60 minutes of 1.19cm water (PCV, 1190% of initial value) and 1.73cm water (SIV, 468% of prechange value). Again central venous pressure changes proportionally more than DA or SIV, however the observed increase is unusual as the majority of animals had substantially decreased PCV pressures by this time.

This figure displays the most common arrangement of pressures observed as SIV pressure was generally consistently higher than that of the PCV no matter the treatment, however at 30 minutes PCV pressure appears higher, a common artifact of cannula occlusion.

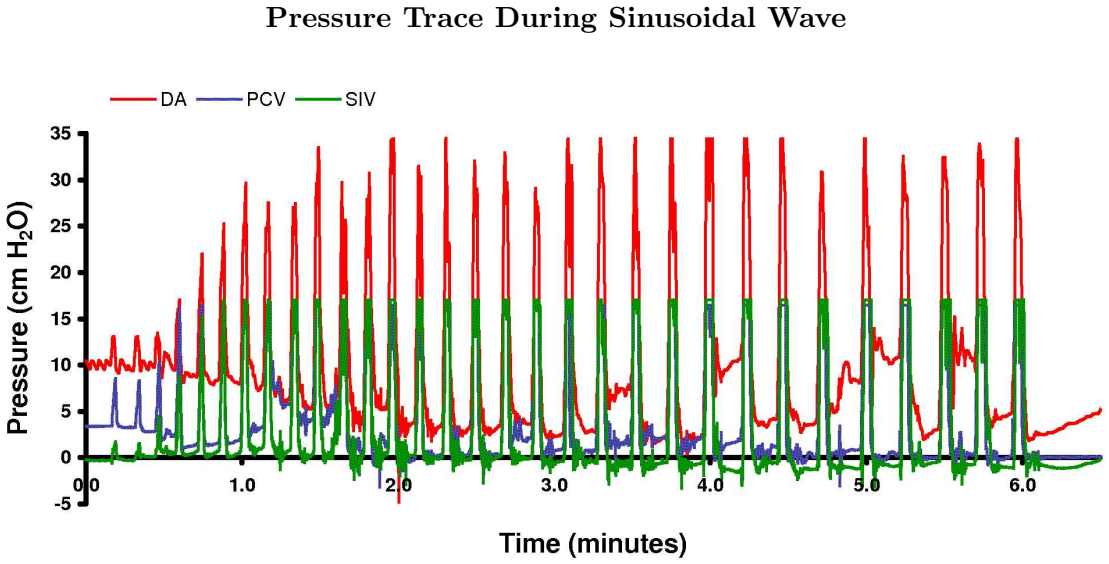


Figure 2.3: Representative trace of blood pressures recorded in the DA, PCV and SIV during sinusoidal wave behavior in one fish. This event occurred 39 minutes after the animal was exposed to 110% seawater to induce volume depletion. The spikes in pressure produced by this behavior were so large (as can be seen from the graph) that the top of the spikes exceed the range setting of the transducer recording the pressures and the signal is attenuated. Note that the blue PCV trace is obscured by the green SIV trace as pressures during spikes were equivalent in these vessels.

In addition to the pressure artifacts caused by swimming behaviors, spikes in blood pressure were also observed regularly. These episodes did not occur whilst the fish was swimming, but they did occur at the same time that muscular sinusoidal waves were seen to pass the length of animal's bodies. Fish did not swim during this behavior but tended to lie uncoiled or partially coiled on the tank floor.

These episodes occurred 23 times out of 127 pressure recording sessions of variable length in which animals were exposed to various treatments. Some episodes occurred more than once in the same fish, as the 23 episodes were seen in only 12 fish. Of the 23 events, 7 occurred shortly after a change of the external medium to induce a volume depleted state (i.e. initial change from 100% to 110% seawater or switch back to 100% after 24 hours or more in 90% seawater) and 3 occurred on the second day of volume depletion. In addition to this, 2 episodes were seen in a fish immediately after administration of phentolamine and thymoxamine (both potential vasodilators phentolamine= non-selective alpha-adrenergic antagonist and thymoxamine= alpha 1 receptor antagonist). So out of 23 recorded events, 12 were observed in animals potentially facing volume depletion. Only 3 episodes were observed animals undergoing volume loading and none occurred 24 hours after volume loading. Five events occurred in animals prior to any volume manipulation treatment and 3 events occurred at least 2 days after animals had undergone any treatment. DA pressure was measured in the 10 minutes preceding the sinusoidal event, the average for all fish exhibiting the behavior was found to be 11.65cm H_2O. This is low when compared

to average resting values taken from 17 fish (see Table 2.1). Average DA pressure in the same animals measured 10 minutes following these episodes was even lower with a value of 8.63cm H_2O.

The effect of these episodes on blood pressure was to produce huge simultaneous spikes in blood pressure of all three vessels during the sinusoidal event. As can be seen in figure 2.3, spikes in pressure during these episodes commonly exceeded the range setting of the pressure transducers. Because of this we do not know what the maximum pressures were during these spikes, but we do know that they could exceed 15cm H_2O in the veins and 35cm H_2O in the DA.

The average length of these episodes was 4 minutes and 22 seconds. The lengths ranged from 25 seconds to 8 minutes and 48 seconds. The rate of spiking appears to slow towards the end of the event and is more prominent when the episodes are longer in duration.

2.3.2 Cardiovascular Parameters in cm H_2O at Rest and During Volume Manipulation

Average Absolute Values Of Cardiovascular Parameters			
Cardiovascular Parameter	**Average Resting Values**	**Loading 60 minutes**	**Depletion 60 minutes**
DA Pressure cm H_2O	14.09±0.46 n=17	*15.80±0.76 n=9 ↑	*12.61±0.71 n=8 ↓
PCV Pressure cm H_2O	0.39±0.08 n=15	*0.20±0.05 n=10 ↓	0.30±0.13 n=5
SIV Pressure cm H_2O	0.67±0.14 n=12	*1.12±0.28 n=6 ↑	*0.24±0.07 n=6 ↓
Heart Rate beats per minute	23.84±1.18 n=15	*24.78±1.75 n=9 ↑	24.5±1.47 n=6
DA Pulse Pressure cm H_2O	1.78±0.18 n=13	*2.80±0.17 n=7 ↑	*1.15±0.27 n=6 ↓

Table 2.1: Average cardiovascular parameters were calculated for each fish at rest and at 60 minutes after induction of volume manipulation, then values for n groups were averaged to calculate the data presented in this table. Average resting values were calculated from measurements made prior to change of the external media. Pre-change values from both loading and depletion groups were compiled to give average resting values. Values are expressed as Mean ±S.E.M. * indicates values significantly different to the appropriate matching resting values for each group (Paired T test *$P<0.05$, arrows indicate direction of change ↑= increase from resting, ↓= decrease from resting).

DA Pressure

The resting range of DA pressure was 10.65cm H_2O to 19cm H_2O (n=17). There is a significant increase and decrease in absolute DA pressure by 60 minutes volume loading and depletion respectively. By 60 minutes into volume loading the average pressure has increased by 1.71cm H_2O while during depletion it has decreased by 1.48cm H_2O.

Venous Pressures

The resting range of pressures for the veins was more variable (as a proportion of the average resting value) with a minimum of 0.1cm H_2O and a maximum of 1.27cm H_2O measured in the PCV (n=15) and a range of 0.23 to 1.62 (n=13 Std. Dev.=0.47) for the SIV.

The range of resting pressures measured in the PCV was not as representative of the normal spread of values as seen in the SIV. The value of 1.27cm H_2O measured as the average resting PCV pressure of one fish is unusually high, with the next highest value being approximately half of this at 0.66cm H_2O. If this unusually high resting PCV pressure is excluded, a new average resting PCV pressure of 0.32cm H_20 is calculated. This value is little different to the mean value of 0.39cm H_2O calculated when it is included.

Notably the absolute PCV pressure had significantly decreased (by 0.2cm H_2O) to approximately half of its resting value by 60 minutes volume loading. SIV pressure on the other hand increased significantly (by 0.45cm H_2O) over the same period for the same treatment. With volume depletion both veins displayed a decrease in pressure although the decrease was only significant in the SIV. PCV pressure was seen to decrease by 0.09cm H_2O while SIV decreased by 0.43cm H_2O.

There are some differences in statistical significance reported in the results section. These differences can be attributed to statistical analyses being applied to both absolute and transformed (percentage) data.

Heart Rate

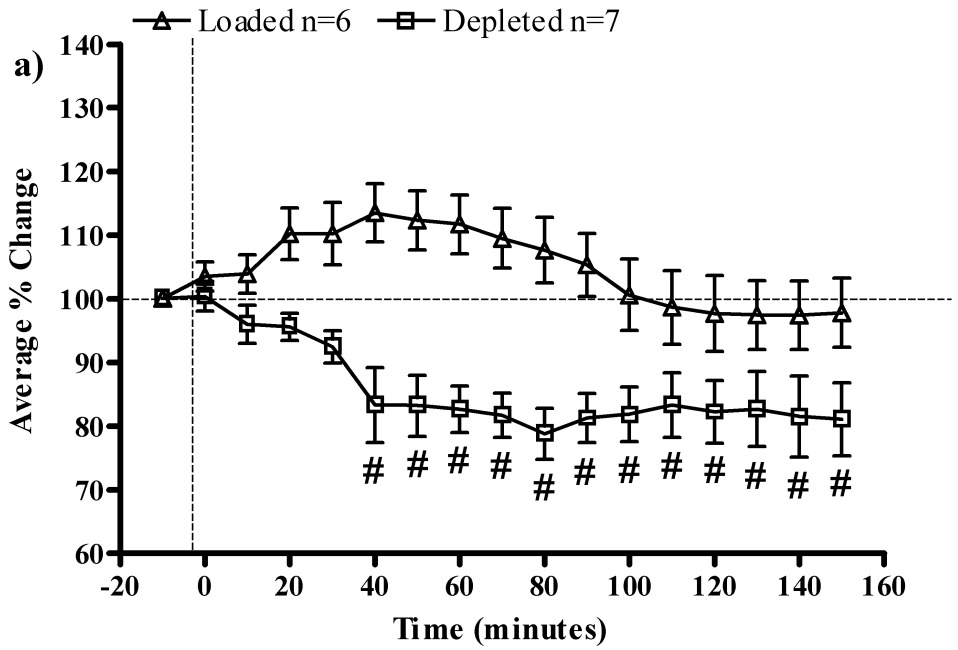
Resting heart rate ranged from 17.4 to 30.9 beats per minute in 15 animals. Only volume loading to 60 minutes resulted in a significantly changed heart rate, showing a small increase compared to pre-change values. By 60 minutes of volume loading, heart rate was an average of 0.9 beats per minute higher. By 60 minutes of volume depletion mean heart rate had increased by 0.7 beats per minute, however this change was not significant.

Pulse Pressure

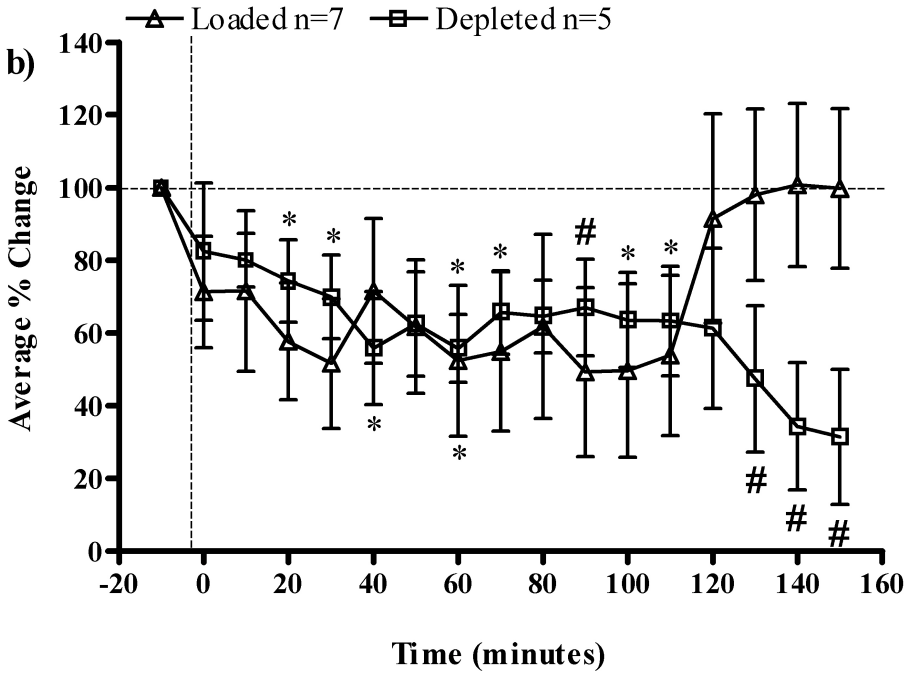
The resting range of pulse pressure in the dorsal aorta was 0.21 to 2.84cm H_2O (n=13). Pulse pressure was seen to change significantly in both volume loading and volume depletion to 60 minutes. On loading an average increase of 1.02 cm H_2O was observed whilst on depletion there was a decrease of 0.63 cm H_20.

2.3.3 Change In Cardiovascular Parameters during Volume Loading And Depletion

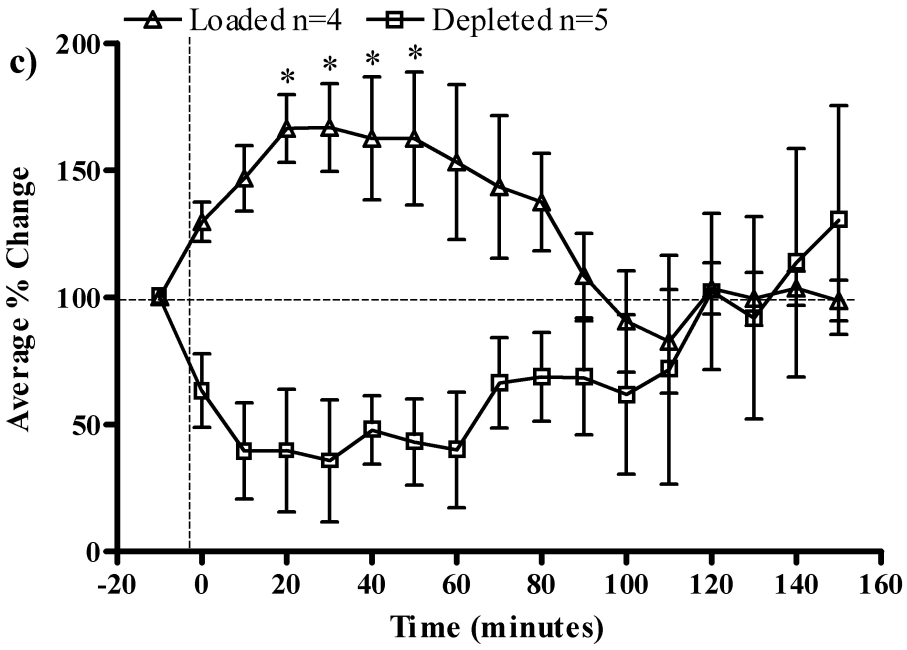
DA Pressure



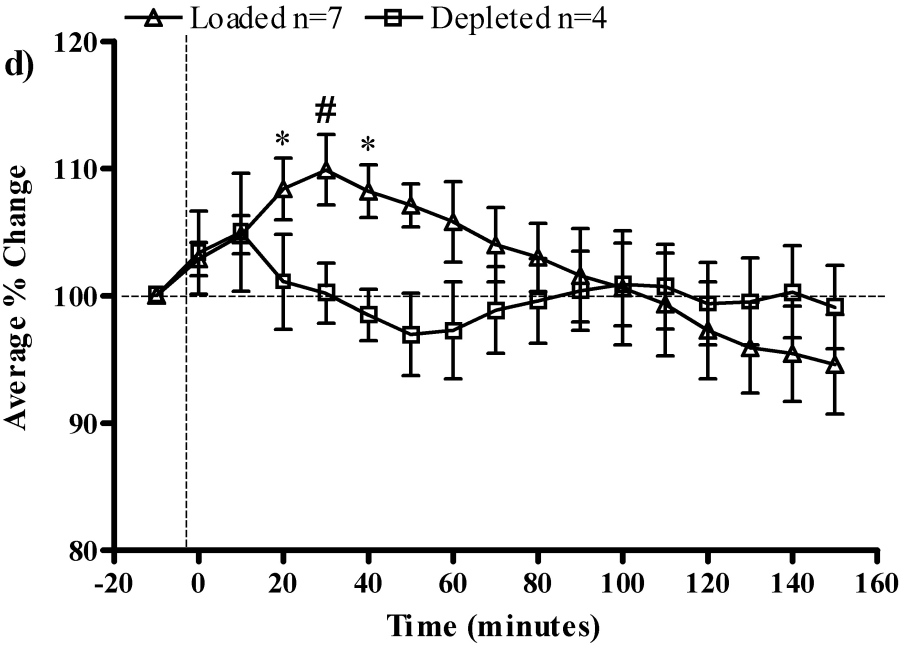
PCV Pressure



SIV Pressure



Heart Rate



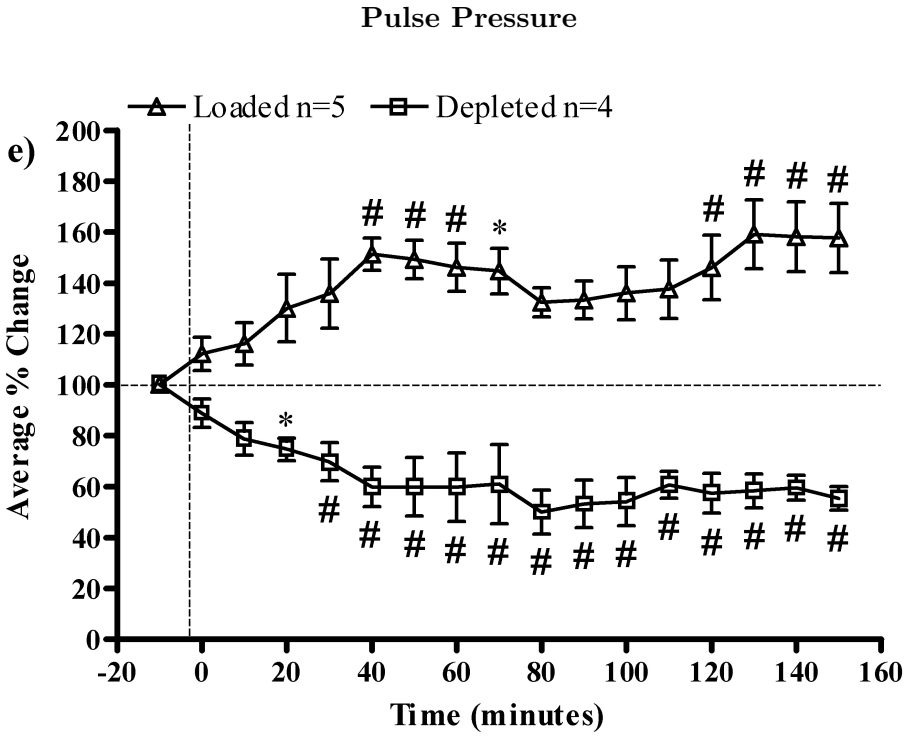


Figure 2.4: All measured cardiovascular parameters during volume loading and depletion. Data are expressed as the average percentage change from resting values ±S.E.M. The medium bathing the animals was changed to 90% (loading) or 110% (depletion) seawater 1-2 minutes prior to time 0 (vertical dashed line). The horizontal dashed line indicates resting values. Repeated Measures Analysis of Variance (ANOVA) with Dunnett's post test were performed to detect significant differences from resting values ($^{\#}$=P<0.01, *=P<0.05, see Tables 2.2 & 2.3 this chapter). Linear trend post tests were also performed (also see Tables 2.2 & 2.3, this chapter). Note; symbols are placed below bars for volume depletion data sets and above bars for volume loading data sets.

Average % Change in DA Pressure

Volume loading induced a transient (though not significant) increase in DA pressure, peaking at 40 minutes. This increase peaks obtains a maximum value of approximately 110%, an increase over resting values of approximately 10%. The overall increase in DA pressure is not significant when Repeated Measures ANOVA are applied to the data spanning 150 minutes. When ANOVA is applied to shortened data sets the increase in pressure does become significant as do trends in other data sets that are not significant over the longer time span (see Tables 2.2 & 2.3). By 100 minutes DA pressure is back to resting levels and is maintained at this level (or slightly lower) to 150 minutes.

With volume depletion DA pressure rapidly decreases becoming significantly lower than resting values at 40 minutes and remaining significantly decreased to 150 minutes. By 40 minutes pressure has decreased to 85% of resting values and remains at this level for the duration of the experiment with no recovery of pressure towards resting values as observed during volume loading.

Average % Change in PCV Pressure

Pressure in the PCV is the only parameter not to display a transient increase early in volume loading. In fact PCV pressure decreases, the opposite to what would be expected should there be no active regulation of venous vascular tone in these animals. PCV pressure decreases to approximately 50% (at its lowest point) of initial values. PCV pressure decreases and the change is significant at 20, 30, 60, 70, 90, 100 and 110 minutes (when ANOVA is applied, overall 60 and 90 minute decreases are also significant see Table 2.3). Interestingly the linear trend (Table 2.3) indicates a significant positive slope overall, due to a recovery back to resting values from 120 minutes.

With the induction of volume depletion PCV pressure decreases consistently, first becoming significant at 40 minutes where the pressure plateaus until 120 minutes where it again begins to decrease. No recovery back to resting pressures is seen during volume depletion and pressures become extremely low, less than thirty percent of resting values at 150 minutes.

Average % Change in SIV Pressure

SIV pressures mirror events occurring in the DA during volume loading, however changes in the SIV are proportionally much larger than those seen in the DA. The SIV displays a significant transient increase in pressure peaking around 30 minutes at approximately 160% of resting values. This is followed by pressure decrease until at 100 minutes pressure has slightly undershot resting values. Between 100 and 150 minutes pressures return to and stay at resting values.

Volume depletion is followed by a rapid decrease in pressure in the SIV which stabilizes at 10 min. Following this from 60 minutes, pressure increases, over-shooting resting values to approximately 125% at 150 minutes. Although this trend is not significant, SIV is the only vessel in which some recovery in pressure is seen during volume depletion.

Average % Change in Heart Rate

Heart rate increases significantly early in volume loading, peaking at 30min with a value of approximately 110%. From this point heart rate is seen to decrease until at 150min it is at approximately 95% of resting values.

With volume depletion heart rate reached its lowest mean value at 50 minutes, however overall there is very little change from resting values.

Average % Change in DA Pulse Pressure

Changes in DA pulse pressure are very clear, pressure significantly increases on loading and decreases on depletion. No recovery towards resting values is observed.

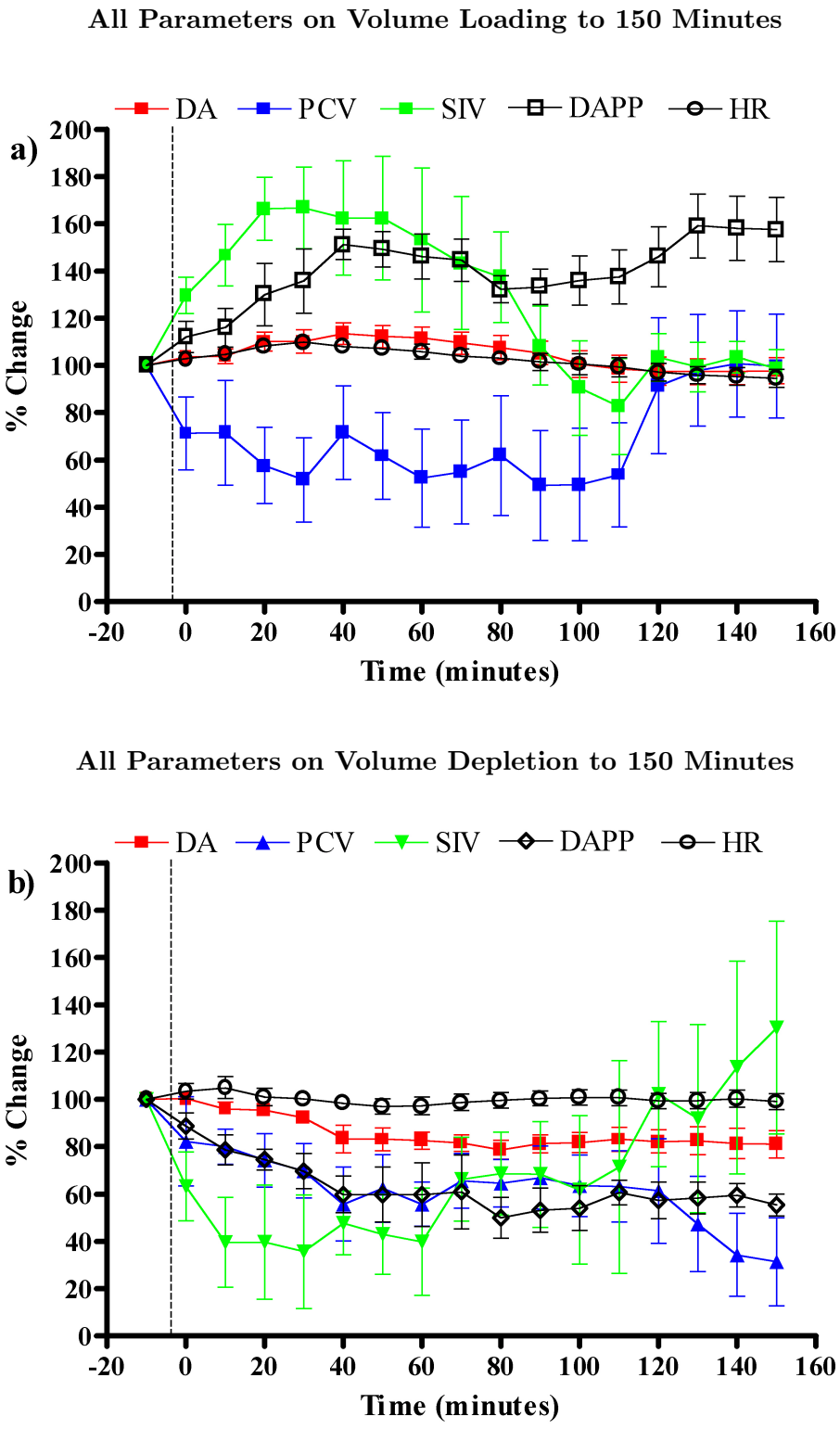


Figure 2.5: Data from Figure 2.4 are displayed to highlight how changes in different cardiovascular parameters during each opposing volume manipulation relate to each other over time. DA= DA pressure, PCV= PCV pressure, SIV= SIV pressure, DAPP= DA pulse pressure and HR= heart rate.

It can be seen from both graphs in figure 2.5 that the greatest proportional changes are seen in the veins followed by DA pulse pressure and that proportionally DA pressure is maintained closer to pre-change values. The two parameters that appear to mirror each other most closely are dorsal aortic pressure and heart rate, as changes in one parameter match changes occurring in the other. For example both of these parameters cross the 100% no change from resting line at the same point in time (100 minutes) during loading.

During volume loading all parameters except DA pulse pressure show some recovery towards resting values. Central venous pressure is the only parameter not to show at least an initial transient increase. It is seen to decrease immediately on exposure to 90% seawater. All of the cardiovascular parameters that do return to pre-change values appear to reach this level at about the same time even though the time points at which they peak and their recovery begins are slightly different.

With loading, the transient increases in parameters peak at similar times, 30-40 minutes post change, which is the time at which osmolarity is seen to stabilize (see figure **??**).

In contrast, SIV pressure is the only parameter in which pressure increases following the rapid decline with volume depletion, although over 150minutes these changes are not seen to be significant. All other parameters decrease to varying degrees over time except for heart rate which is maintained at resting values and does not significantly change over the 150 minute monitoring period. With depletion SIV pressure is the parameter which decreases most rapidly on exposure to 110% seawater and proportionally decreases the most.

Cardiovascular Parameters During Volume Depletion; Statistical Analyses												
Length of Data Set	**60 minutes**				**90 minutes**				**150 minutes**			
Statistical Test	**ANOVA**		**Linear Trend**		**ANOVA**		**Linear Trend**		**ANOVA**		**Linear Trend**	
	Significant	**n**	**Significant**	**Slope**	**Significant**	**n**	**Significant**	**Slope**	**Significant**	**n**	**Significant**	**Slope**
DA Pressure	Yes P<0.0001	8	Yes P<0.0001	-1.42	Yes P<0.0001	7	Yes P<0.0001	-2.35	Yes P<0.0001	7	Yes P<0.0001	-1.18
PCV Pressure	Yes P=0.0014	6	Yes P<0.0001	-3.40	Yes P=0.0067	5	Yes P=0.0002	-2.95	Yes P=0.003	5	Yes P<0.0001	-2.77
SIV Pressure	No	6	Yes P=0.0124	-2.77	No	5	No	-0.44	No	5	Yes P=0.0031	3.65
Heart Rate	Yes P=0.0035	6	Yes P=0.0002	-0.51	No	5	Yes P=0.0010	-0.62	No	4	No	-0.13
DA Pulse Pressure	Yes P<0.0001	6	Yes P<0.0001	-3.35	Yes P<0.0001	5	Yes P<0.0001	-4.33	Yes P<0.0001	4	Yes P<0.0001	-2.08

Table 2.2: Statistical analyses of cardiovascular parameters during volume depletion; statistics applied over 60,90 and 150 minute data sets.

Cardiovascular Parameters During Volume Loading; Statistical Analyses												
Length of Data Set	**60 minutes**				**90 minutes**				**150 minutes**			
Statistical Test	**ANOVA**		**Linear Trend**		**ANOVA**		**Linear Trend**		**ANOVA**		**Linear Trend**	
	Significant	**n**	**Significant**	**Slope**	**Significant**	**n**	**Significant**	**Slope**	**Significant**	**n**	**Significant**	**Slope**
DA Pressure	Yes P<0.0001	9	Yes P<0.0001	1.22	Yes P=.0015	7	Yes P=0.0074	0.65	Yes P=0.0064	6	Yes P=0.0010	-0.65
PCV Pressure	Yes P=0.0010	10	Yes P<0.0001	-2.25	Yes P=0.0213	8	Yes 0.0005	-3.15	Yes P<0.0001	7	Yes P=0.0291	1.13
SIV Pressure	No	6	Yes P=0.0025	2.58	Yes P=0.0074	4	No	0.29	Yes P<0.0001	4	Yes P<0.0001	-3.60
Heart Rate	Yes P<0.0001	9	Yes P<0.0001	0.41	Yes P=0.0002	7	No	-0.02	Yes P<0.0001	7	Yes P<0.0001	-0.69
DA Pulse Pressure	Yes P<0.0001	7	Yes P<0.0001	5.93	Yes P<0.0001	5	Yes P<0.0001	3.44	Yes P=0.0004	5	Yes P<0.0001	2.54

Table 2.3: Statistical analyses of cardiovascular parameters during volume loading; statistics applied over 60,90 and 150 minute data sets.

Table 2.2 & 2.3

Results of repeated measures ANOVA and linear trend post tests applied to the average percent change from resting data over 60, 90 and 150 minutes. The 150 minute data set is the same as used to generate figure 2.4 (this chapter). Note that n numbers are greater for the 60 minute and 90 minute data sets, as extra data from additional animals were available over these shorter collection periods. This is due to the difficulty of collecting data over extended periods of time.

DA Pressures

During depletion, Repeated Measures ANOVA applied to all three data sets indicate that DA pressure is changing significantly with time. Linear trend shows significant downward trends in all three data sets demonstrating decreases in DA pressure.

The situation in the same vessel during volume loading is quite different. Values are still all significantly different from resting however linear trend tells an interesting story. Over 60 minutes there is a significant increase in DA pressure with a positive slope of 1.219. Over 90 minutes a significant increase is still seen, however the slope has been reduced to 0.6461. Over 150 minutes the overall linear trend is negative with a value of -0.6498 demonstrating a significant decrease in DA pressure rather than an increase as would be expected if no regulation was occurring.

PCV Pressures

Central venous pressures mirror events occurring in the DA during volume depletion. ANOVA indicates values change significantly with time and linear trend shows the changes to be significant decreases in PCV pressure in all three data sets.

Again, the most interesting events are seen during volume loading. ANOVA indicates significant changes over time in all data sets, with slopes of -2.251 and -3.146 for the 60min and 90min data sets respectively. Again, decreases in pressure are seen during volume loading instead of increases as may be expected. When the statistical tests are applied over 150 minutes, the results are significant, however the trend has become positive with a slope of 1.127, representative of the rapid recovery seen later on in this data set.

SIV Pressures

When ANOVA is applied to SIV data sets over these time periods with volume depletion, the overall changes are not significant. However, when the post test for linear trend is applied, significant linear trends can be identified. Over 60 minutes a significant negative slope of -2.772 occurs associated with the initial rapid decrease seen in SIV pressure. When linear trend is examined over 90 minutes no significant trend is observed indicating a flattening out of pressure over this time. By 150 minutes the trend has again changed,

to a significant positive slope of 3.645 associated with the increase in pressure observed after 100 minutes.

During volume loading overall changes in SIV pressure are not significant in the 60 minute data set, however they are over the 90 and 150 minute data sets.

SIV pressure initially shows an increase with a positive slope value of 2.582 over 60 minutes. Over 90 minutes, although values have changed significantly overall from resting, there is no significant trend in slope. Over the 150 minute data set the slope is highly significant and negative, with a value of -3.602 highlighting the rapid recovery to resting values that occurs late in this data set.

Heart Rate

When ANOVA is applied to the shortest data set during depletion a significant change is detected. The slope is significant and slightly negative at -0.513 showing a very slight initial decrease in heart rate with volume depletion. The overall change is negated when ANOVA is applied to the 90 and 150 minute data sets, although over 90 minutes there is still a significant linear trend of -0.618. By 150 min neither ANOVA nor linear trend are significant indicating heart rate is maintained at resting values.

Heart rate changes significantly with time over all three data sets during volume loading. To begin with, over 60 minutes, an increase in heart rate is observed (slope 0.4123). When looked at over 90 minutes no significant linear trend can be detected as heart rate peaked at 30 minutes and has declined, but not below resting values. By 150 minutes a significant negative linear trend becomes apparent (slope -0.6939) as heart rate decreases below resting after 100 minutes.

Pulse Pressure

During depletion, a consistent significant decline is observed as all three data sets display significant changes over time with negative slopes of -3.349, -4.331 and -2.077 respectively.

During loading pulse pressure shows significant increases with time in all three data sets with positive slope values of 5.930, 3.438 and 2.538 for 60,90 and 150 minutes respectively, indicating the rate of increase is slowing with time.

2.3.4 Cardiovascular Parameters After One Day of Volume Depletion

Volume Depleted (110%) Sea Water				
Parameter	**Manipulation**	**Number of Animals**	**Average Values**	**Significance**
DA Pressure	Resting	n=8	13.58 ± 0.20	Yes P<0.0001
DA Pressure	Next Day	n=8	11.78 ± 0.17	
PCV Pressure	Resting	n=6	0.13 ± 0.05	No* P=0.0515
PCV Pressure	Next Day	n=6	0.02 ± 0.03	
SIV Pressure	Resting	n=6	0.47 ± 0.04	Yes P=0.0027
SIV Pressure	Next Day	n=6	0.25 ± 0.05	
Heart Rate	Resting	n=7	21.43 ± 0.58	No P=0.6964
Heart Rate	Next Day	n=7	21.11 ± 0.55	
DA Pulse Pressure	Resting	n=7	1.91 ± 0.03	Yes* P=0.0010
DA Pulse Pressure	Next Day	n=7	2.21 ± 0.07	

Table 2.4: Comparison of resting values to 24 hour values, after this period of volume depletion.

Volume Loaded (90%) Sea Water				
Parameter	**Manipulation**	**Number of Animals**	**Average Values**	**Significance**
DA Pressure	Resting	n=6	14.31 ± 0.33	Yes* P=0.0478
DA Pressure	Next Day	n=6	13.50 ± 0.16	
PCV Pressure	Resting	n=7	0.36 ± 0.01	No* P=0.0572
PCV Pressure	Next Day	n=7	0.50 ± 0.07	
SIV Pressure	Resting	n=6	0.68 ± 0.05	No P=0.3493
SIV Pressure	Next Day	n=6	0.61 ± 0.05	
Heart Rate	Resting	n=5	21.85 ± 0.27	Yes P<0.0001
Heart Rate	Next Day	n=5	17.94 ± 0.28	
DA Pulse Pressure	Resting	n=5	1.98 ± 0.04	No* P=0.1918
DA Pulse Pressure	Next Day	n=5	1.89 ± 0.06	

Table 2.5: Comparison of resting values to 24 hour values, after this period of volume loading.

Table 2.4 & 2.5

Comparison of resting values with values collected the next day from the same animals exposed to either volume loading or depletion for 24 hours. All pressure values are expressed as cm H_2O and heart rate is expressed as beats per minute. Values marked with * indicate P values calculated using Welch's correction due to groups having significantly different variances.(Unpaired T tests, $P<0.05$).

DA Pressure

DA pressure was significantly changed in both volume depleted and loaded animals at 24 hours after external media change. DA pressure was markedly decreased by 24 hours on both depletion and loading. The decrease was greater on depletion than loading with an average decrease of 1.8 cm H_2O after 24 hours of depletion and a decrease of 0.81 cm H_2O after 24 hours of volume loading

PCV Pressure

24 hours after volume depletion PCV pressure was decreased but this was not significant However, the P statistic did approach significance with a value of 0.0515.

There was also no significant difference in pressure between resting and 24 hour values for volume loading. Although again, the P statistic approached significance with a value of 0.0572 and PCV pressure had nominally increased.

SIV Pressure

24 hours after volume depletion, SIV pressure had significantly decreased compared to resting values. No significant difference from resting values was detectable after 24 hours of volume loading, indicating that only with loading were the animals able to return to resting values.

Heart Rate

Heart rate had not changed after 150 minutes of volume depletion (Table 2.2) and was still maintained at resting values at 24 hours. No significant difference was detectable and there were only 0.32 beats per minute difference between the resting and 24 hour volume depletion values.

Interestingly, similar to the 150 minute data set, heart rate was still markedly and significantly decreased at 24 hours of volume loading. Heart rate had decreased by nearly 4 beats per minute (3.91), which is 17.9 % lower than the resting value.

Pulse Pressure

Pulse pressure was the only cardiovascular parameter to show a significant increase 24 hours after the induction of volume depletion. This increase at 24 hours is interesting as in the short term (60, 90 and 150 minutes) substantial and significant decreases in pulse pressure were observed.

By 24 hours of volume loading, there was no significant difference in pulse pressure when compared with resting values. In the short term highly significant increases were observed (Table 2.3) but these have been negated after 24 hours of volume loading.

2.4 Discussion

2.4.1 Changes in Plasma Osmolarity With Volume Manipulation

There are rapid and significant changes in hagfishes' plasma osmolarity upon change of the external media to ∼110% and ∼90% sea water during volume depletion and loading treatments respectively. After an equilibration period of approximately 60 minutes, plasma osmolarities were not notably different from the external medium and by 24 hours exposure, plasma osmolarities still matched that of the external medium. Our results support the findings of other researchers who have found hagfish to behave as osmoconformers (McFarland and Munz 1958; Cholette et al. 1970; Hardisty 1979; Toop and Evans 1993). Our measurements do not indicate plasma osmolarities differ significantly from that of the external medium. We have confirmed that *E. cirrhatus* osmoconforms over the range 90-110% sea water.

Although we know plasma osmolarity matched that of the external medium, we did not analyse the plasma for individual ionic components. Authors have reported plasma sodium concentrations to be higher than that found in the environment by 10% to 19% (Robertson 1954; Bellamy and Chester-Jones 1961; McFarland and Munz 1965; Morris 1965). It is unlikely that this is driven by kidney function as hagfish produce a nearly isotonic urine and are unable to reabsorb significant amounts of sodium via renal mechanisms (McFarland and Munz 1965; Morris 1965; Fels et al. 1989). Bartels (1998) argues that specific mitochondria rich cells found in the gill epithelia may be responsible for raised plasma sodium, as these cells possess pronounced ultrastructural characteristics of ion transporting cells. The exact role of these cells has yet to be resolved. Interestingly, in an earlier study McFarland and Munz (1958) found that when blood sampling *Eptatretus stouti* higher osmotic pressures were obtained from the second animals to be sampled in comparison to the first regardless of the osmolarity of the external medium (85.5%, 100% & 116.1% seawater for 30 hours). In the 1958 study, animals were housed together in the experimental medium. It is likely that high plasma osmolarity readings were due to a stress response, as the second fish to be sampled had been disturbed as the first fish was removed from the tank. This becomes even more likely when it is considered that the third set of animals were left undisturbed for 1 hour prior to sampling and their osmolarities were more comparable to the first set of animals. Plasma osmolarities from the third set of animals were iso-osmotic to the external medium. We took extreme care to avoid disturbing the animals during blood sampling and our data do not indicate plasma as being hyperosmotic to the medium.

Such rapid changes in plasma osmolarity as seen in this study, demonstrate that hagfishes are indeed permeable to water and salts. This work suggests that hagfishes began to be challenged volumetrically almost immediately upon the induction of volume manipulation, with maximum volume disturbance occurring after 60 minutes.

Toop and Evans (1993) exposed the Atlantic hagfish *Myxine glutinosa* to acute changes in external osmolarity, changing the media to 85% or 115% sea water for a period of

13 days. They found that maximum weight changes and therefore volume changes had occurred by 6 hours. Toop and Evans did not weigh their animals in the intervening time between induction and 6 hours post induction and in light of our results, we suggest that maximum weight/volume change occurs much earlier.

Hagfishes as a whole have very little capacity to withstand osmotic challenges and under normal conditions are considered to be stenohaline. We observed that *E. cirrhatus* will tolerate acute changes for short periods of time and the literature suggests that they withstand gradual changes for slightly longer periods (Cholette et al. 1970). Acute exposure to unfavourable osmotic conditions over extended periods of time is beyond the ability of these animals to cope. Under these conditions animals become moribund and death follows if conditions are not improved (personal observation).

The Atlantic hagfish *M. glutinosa* has been shown to survive in salinities ranging from 57% to 130% sea water providing the daily concentration change does not exceed 15mOsm (Cholette et al. 1970). McFarland and Munz (1965) exposed the Pacific hagfish *Eptatretus stouti*, over a period of 7 days to a range of low and high salinity waters (40-122% sea water) followed by a 7 day return to 100% seawater, which the animals tolerated.

In our study, *Eptatretus cirrhatus* were exposed to an acute change of 100mOsm and as a result, many exhibited a decline in condition by 24 hours exposure, this was particularly evident in animals that underwent volume depletion.

It is probable that interspecies differences exist in hagfishes' ability to tolerate salinity change. Although it is possible other species (i.e. *M. glutinosa* and *E. stouti*) may be better able to tolerate salinity change than *E. cirrhatus*, it is unexpected, as *E. cirrhatus* is the most coastal of these species. In New Zealand *E. cirrhatus* is found in coastal regions often at depths of less than 100m. In this environment they are likely to experience more variability in environmental salinity than the other species which are commonly found in deep water (Strahan 1963; Davie et al. 1987).

It is most likely that in this study, the combination of acute salinity change and chronic cannulation of large blood vessels compromised the animals ability to tolerate the stress of osmotic challenge. After 24 hours acute exposure, the animals were returned to normal seawater. Upon the switch back to normal seawater, the animals' condition could be seen to improve immediately and 24 hours following this, the majority of animals had made a full recovery and were exhibiting normal behaviour.

Both Toop and Evans (1993) and McFarland and Munz (1965) observed that hagfish remaining in altered media were able to regulate their weight/volume back towards normal pre-change levels over a period of days when volume loaded, but were unable to regain the weight lost when volume depleted. *M. glutinosa* gained a maximum of 8% of their initial weight by 6 hours in 85% sea water, by 36 hours weight was still increased by 2-4% and it wasn't until 48 hours that weights were no longer statistically different from starting values (Toop and Evans 1993). *E. stouti* increased in weight by a maximum of 10% in 80% sea water and did not approach normal weights until 5 days in this medium (McFarland and Munz 1965).

Quite how these animals were able to "volume regulate" when loaded has not been elucidated, but one possible mechanism could be the excretion of the excess volume via renal mechanisms. Excretion via renal mechanisms is likely to have had little if any impact on volume regulation during the short time span over which we observed cardiovascular parameters (the first 150 minutes and possibly 24hour measurements). This is due to the limited filtration capacity of the agnathan kidney (Fels et al. 1998; Riegel 1998) and is supported by the fact that previous studies have shown that it takes a number of days for fish to rid themselves of the excess volume.

The work of Toop and Evans (1993) has also provided evidence that change of the external medium has direct effects on the blood volume as haematocrits increased and decreased significantly on volume depletion and loading respectively. Although we did not measure haematocrits during volume manipulation, it is not unreasonable to assume the same events occurred in our study as we observed more dilute blood samples that were easier to obtain during loading (personal observation).

In summary, *E. cirrhatus* does not appear to have significant osmoregulatory ability when exposed to an acute osmotic challenge of 10% change. The ∼10% change in osmolarity and volume evoked significant physiological responses in the cardiovascular system as can be seen from the cardiovascular parameters illustrated in figure 2.4. Our findings suggest that some changes in the measured cardiovascular parameters are responses to direct effects of acute volume change on blood volume. Our work suggests the observed compensations in the short term, are due to changes in the cardiovascular system and are unlikely to be due to compensation by other physiological systems.

2.4.2 Examples of Pressure Recordings and Associated Artefacts

Volume Loading and Depletion

Figure 2.2a and 2.2b are examples of raw traces from one fish, recorded during volume depletion and volume loading experiments. These figures show some typical and atypical features that could be observed during pressure recording sessions.

These figures show that pressures in all three vessels had changed rapidly post volume manipulation. Volume depletion (figure 2.2a) caused pressures in all three vessels to decrease, after a large movement artefact at 10 minutes and it can be seen that with volume loading, pressures in all vessels had increased.

However the increase in PCV pressure with volume loading was atypical as more commonly central venous pressures decreased with volume loading (see figure 2.4). The increase in PCV pressure after 10 minutes in the volume depletion trace is an example of an artefact, the presence of an air bubble or blockage of the cannula, occasionally observed when recording pressures. These artefacts can be easily identified as pressures are not stable and tend to fluctuate as can be seen from the PCV trace in figure 2.2b. Additionally there are no corresponding fluctuations in recordings from other vessels which suggests this is not a movement artefact. Air bubbles and blockages could sometimes be

removed by flushing the cannula with 0.4ml of heparinised hagfish saline (a volume that in preliminary experiments had no effect on intravascular pressures). Blockages that could not be removed resulted in unstable pressure recordings that were excluded from analysis. Interestingly, the PCV trace was stable at 10 minutes which suggests PCV pressure was increased in this fish at this time. This is not in agreement with the trend in figure 2.2b where the average pressure for 5 fish has decreased to 80% of the resting value at this time point (although this change is not significant). This is not surprising however as there may be a greater variation in responses immediately after induction of volume loading, as fish may be affected by volume change at slightly different rates. This could also indicate the presence of a lag period between the time it takes for unfavourable changes in pressure to occur or be detected and the time for compensatory systems (whether passive or active) to take effect.

Effects of Activity

Figure 2.2a) illustrates the effects of activity of the animal upon intravascular pressures. Occasionally when animals were disturbed (for example when cannulae were attached to pressure recording devices) animals would react and begin to swim or would exhibit burrowing behaviour. Physical activity induced pressure in all three vessels to increase substantially and remain increased for the duration of the activity. The impact of this phenomenon on the cardiovascular system as a whole is unclear, although it is not unreasonable to assume an increase in cardiac output probably occurs at this time due to increased venous return. It can be seen that arterial pressures have increased by approximately 4 cm H_2O for the duration of this event even though the fish is being exposed to volume depletion.

Other researchers have reported similar pressure effects in other fish species related to physical activity. In work on the Port Jackson shark, Satchell (1967) reported increases in caudal vein pressure which reflected the timing and intensity of lateral flexion of the trunk. Physical activity could be acting to increase the aerobic capacity of the generally sluggish hagfish by mobilizing blood and increasing circulation. The ability to mobilize blood volume simply through physical movement could provide an adaptive mechanism to "kick start" the animal in times of need i.e. escape from predators, feeding frenzy, recovery from anoxia etc. Axelsson et al (1990) suggest physical activity could be a mechanism to increase cardiac output after periods of anoxia or hypoxia in *M. glutinosa*. It seems likely from our observations that activity does mobilize unexploited blood volume, but the effect of this on the overall aerobic performance of the animal is yet to be determined.

Although it is possible physical activity may in some way aid hagfishes' circulation, some authors suggest that it may in fact compromise cardiovascular performance. Forster (1998) comments that body movements associated with swimming may compromise cardiac function *in vivo* and prevent the animals from achieving the maximum values for cardiac output determined from studies on isolated perfused hearts.

There is also potential for the increases in pressure seen with activity to have the opposite effect, and reduce the effective blood volume by forcing blood out of the central circulation and into the venous sinus system. Blood enters the branchial sinus at papillae on the branchial vessels, but the routes of entry into the subcutaneous sinus are not fully understood (Cole 1912; Elger 1987; Forster 1997). Raised blood pressures could increase the movement of blood through the arterial papillae into the sinus system, with the consequent need for the return of that blood to the central circulation (Forster 1998). The caudal and cardinal hearts of the hagfish are responsible for returning blood within the subcutaneous sinus to the central circulation (Satchell 1984; Forster 1998). It has been observed that the caudal heart of *Myxine* starts to beat soon after the fish stops swimming and it is at this time that caudal heart rate is maximal, possibly indicating an enhanced need to return blood to the central circulation (Satchell 1984). Variations in the haematocrit of subcutaneous sinus blood also suggest a redistribution of blood from the central circulation to the SCS after activity. Due to plasma skimming where blood enters the sinus system, the haematocrit of SCS blood is lower than that of the central circulation in resting animals (Johansen et al. 1962; Forster et al. 1989). After hagfish struggle, the haematocrit of the SCS blood increases, resembling the haematocrit of the central circulation (Johansen 1963).

These ideas may be criticised, as currently very little is known about the partitioning of blood between the central circulation and the sinus system. It is possible that the action of the caudal heart after swimming may not be acting to replace lost volume, but to add extra volume to the central circulation which may aid recovery from exercise. The ability for a rapid return of blood from the sinus system may be questioned as there is a low turn over of blood between the central and sinus compartments, although these measurements were made in resting fish (Forster et al. 1989; Forster 1997; Forster et al. 2001). The higher haematocrits measured in SCS blood after activity may also be explained by resuspension of blood cells as the action of struggling could have this effect. Whatever the effect of activity on *E cirrhatus*'s cardiovascular performance, the intravascular pressure increases were transient and pressures decreased immediately with the cessation of activity.

Acute Volume Change

With the exclusion of the PCV trace in figure 2.2b for aforementioned reasons, the early 30 minute changes in pressure were in agreement with those predicted from salinity manipulations and suggest that in this fish intravascular blood volumes were altered rapidly. There was little evidence of an early regulatory cardiovascular response that influenced the measured cardiovascular parameters at this time, again possibly suggesting a lag phase prior to compensation that was observed during volume loading. Changes in intravascular pressure may indicate changes in intravascular volume and/or changes in regulated parameters such as vascular tone and compliance. As these figures show early pressure changes correlated with the modified media, they suggest changes in intravascular volume

soon after induction of volume manipulation.

Events In the SIV

These figures also demonstrate the most common pattern of venous pressures in that pressure in the SIV was generally higher than in the PCV although pressure in both was commonly less than 1cm H_2O.

Although not shown in the figures, we occasionally measured negative pressures in the SIV. We suspect that in these instances the tip of the cannula had moved very close to or into the portal heart as negative values were observed on the down stroke of the beat of the portal heart. This could be due to some aspirational ability of the portal heart. It is commonly accepted that in order to generate subambient pressures for aspirational filling of the heart, the chambers of the heart need to be surrounded by a rigid pericardium. Although hagfishes lack a rigid pericardium, both their systemic and portal hearts are surrounded by connective tissues. It has been shown in an *in situ* preparation, that cutting the connective tissue surrounding the portal heart significantly decreases CO (Johnsson et al. 1996).

Pressures During Sinusoidal Wave Behaviour

Figure 2.3) is a representative trace from an animal displaying a remarkable behaviour. It was observed in a number of fish that huge simultaneous spikes in blood pressure occurred in all three vessels, at the same time that sinusoidal muscular contractions were seen to pass the length of the fishes bodies.

To my knowledge no other researcher has reported this phenomenon, though this may be attributed to the limited number of studies which have directly measured blood pressures in conscious unrestrained hagfish. Axelsson et al (1990) reported a coughing behaviour in *M. glutinosa*, that appeared as notches in aortic pressure recordings, however these events were of a much smaller magnitude than the pressure spikes reported here. These workers also reported sinusoidal tail movements in the same species after periods of anoxia and hypothesised that this may be a mechanism to increase venous return.

What could the purpose of this behaviour be in *E. cirrhatus* ? Does it have adaptive significance, and if so what?

Interestingly this behaviour only occurred as the animals were resting on the tank floor and not while they were moving or whilst tightly coiled. *E. cirrhatus* is generally found coiled on rocky substrates and is not thought to burrow, however these peristaltic waves resembled burrowing behaviour displayed by *M. glutinosa* (Martini 1998). Martini (1998) describes the burrowing activity of *Myxine* "When preparing to burrow the animal assumes an angle of 45-90 $^\circ$ to the bottom and swims vigorously, driving the head into the substrate. The swimming movements continue as the head moves into the substrate following a sinusoidal path that roughly parallels the surface. When one-third to one-half of the animal has entered the substrate, swimming movements cease. Sinusoidal progression

of the head and anterior trunk continue, punctuated by longitudinal contractions that pull the immobile posterior portion of the animal into the burrow in a series of pulses".

The "longitudinal" pulses that Martini describes, matches the almost convulsive behaviour we observed. However this behaviour did not serve a locomotory function in *E. cirrhatus* as it did not propel the animals in any particular direction. Pulsatile, contractile waves were seen to pass in a posterior to anterior direction and appeared to jolt the anterior portion of the animal. The animals appeared to "cough" as the waves passed over their anterior portion, blowing water out of their nasal duct rather than drawing it in. This has also been described in *Myxine*, as animals burrowed in a muddy substrate have been seen to forcefully blow water out of the nasal duct in a "cough" like manner (Malte and Lomholt 1998).

It is possible that the instinct to burrow in times of stress is retained by *E. cirrhatus* even though this species has not been observed to burrow in its natural environment. This behaviour was correlated with manipulations of animals that upset homeostatic balance. Directly after external media change *E. cirrhatus* was often observed to swim vigorously against the bottom of the tank, pushing at the tank floor in a burrowing like manner. *E. cirrhatus* is also likely to employ burrowing during feeding as hagfish are known to tunnel into the soft viscera of dead or dying prey, a necessity of their jawless morphology.

Whether this activity has any lasting influence on the cardiovascular system is unknown, however, intravascular pressures increase massively and it is likely there is a mobilization of blood volume and possibly a concomitant increase in cardiac output. This said, the same considerations apply that have been previously discussed in relation to the effects of activity on the partitioning of blood between the central circulation and sinus system.

Like the effects of physical activity, the effects of these pressure spikes appeared to be transitory. Pressures were observed to immediately drop after waves of muscular contraction passes the point in the vasculature that the pressure transducers monitor. As mentioned, this could serve to improve the hagfishes circulation. However, it could also redirect blood volume into the venous sinus system. Even if circulation is increased, the benefit gained is uncertain as the effects on ventilation-perfusion matching are unknown. Forster (1998) comments that a delicate balance exists between blood vascular and water duct pressures in the hagfish gill pouch, but that these parameters should be affected equally by activity of the animal as the gill pouches are internalized and both compartments are subject to the same muscular activity. This is supported by the finding that in swimming hagfish, PaO_2 is maintained at high levels (Wells et al. 1986).

It can be concluded that this behaviour was generally evoked by stress, with a bias towards volume depletion. This is evident as 12 out of 23 of these episodes were recorded in animals facing volume depletion versus only 3 episodes recorded in volume loaded animals. Average DA pressure recorded in the 10 minutes preceding the episodes was low, when compared to normal resting values, but still within the resting range. Although this bias exists, there is little evidence to suggest that these episodes raised low intravascular

pressures. The average DA pressure recorded after these events was lower than those recorded before. It is likely that the animals that exhibited this behaviour prior to volume manipulation, were stressed and may not have completely recovered from surgery. It is impossible to estimate the volume condition of these animals, but it is more likely they were depleted rather than loaded due to the possibility of post-surgical haemorrhage depleting blood volume.

Because this behaviour was evoked in an animal that had notably decreased pressures after the administration of phentolamine and thymoxamine (potential vasodilators), it can be directly linked to decreases in intravascular pressures, which in this case were not caused by a change of osmolarity of the external medium or the blood. The average dorsal aortic pressure in this animal 10 minutes prior to drug administration was 12.61cm H_2O, this decreased to 11.98cm H_2O after phentolamine and then to 7.61cm H_2O post thymoxamine. The pressure spikes began 3.45 minutes after thymoxamine was administered. In the 10 minutes after the pressure spikes, DA pressure in this animal had increased slightly to 8.41.

Although the duration of these events varied, it was apparent that the rate of spiking became slower towards the end of the episode, especially when the episodes were longer in duration. It is probable that this slowing in the rate of spiking is due to exhaustion which is likely to be exacerbated by the stressed state of the animal.

2.4.3 Average Cardiovascular Parameters at Rest and During Volume Manipulation

Blood Pressures In *E. cirrhatus*

These values reiterate the findings of other researchers, that hagfishes have the lowest arterial blood pressures recorded in the sub phylum Vertebrata Satchell 1986a; Davie et al. 1987; Forster et al. 1988; Axelsson et al. 1990; Forster et al. 1992). The average resting dorsal aortic pressure of 14.09 cm H_2O presented here, is similar to values reported in the literature for this species. Forster and co-workers (Forster et al. 1992) measured an average DA pressure of 13.26 cm H_20 in resting normoxic *E. cirrhatus.* Forster et al (1988) obtained a resting DA value of 10.91 cm H_20 prior to a bout of swimming, during which DA pressure barely changed to 10.20 cm H_20.

Resting DA pressures measured in other species of fish are considerably greater than in hagfish. Examples of this can be seen in table 1.1 Chapter One, with values of 39.77cm H_20 for the dogfish (*Scyliorhincus canicula*) and 32.63cm H_20 in the Atlantic cod (*Gadus morhua*) (Short et al. 1979; Axelsson and Nilsson 1986).

The values presented in table 2.1 also show *E. cirrhatus* to have extremely low venous pressures, with average resting pressures less than 0.7 cm H_20 in both PCV and SIV. Johnsson et al (1996) recorded similarly low pressures with a value of 40 Pa or 0.41 cm H_2O in the SIV of *E. cirrhatus* (Johnsson et al. 1996).

Low venous pressures have been recorded in other "primitive" species, particularly

elasmobranch fish (Satchell 1961; Satchell 1986b; Satchell 1992). However, resting central venous pressures in other vertebrate species are not consistently as close to zero as those we recorded in conscious *E. cirrhatus,* even when recorded in anaesthetised animals. Interestingly, pressure in the posterior cardinal vein of some elasmobranchs has been shown either to be negative, or to cycle between positive and negative pressures, indicating use of *vis-a-fronte* or aspirational force to aid the filling of the heart (Satchell 1971; Satchell 1986b; Satchell and Weber 1987). *Vis-a-fronte* filling (force from in front) uses the energy of the ventricular contraction to distend the atrium, creating suction and so central venous pressures can become sub-ambient (Farrell 1993).

Venous pressures in teleost fish vary, depending on species and the vessel in which pressure is measured, but pressures are generally higher than those seen in hagfish. An average pressure of 2.85 cm H_2O has been measured in the caudal vein of the relatively inactive benthic species *Platichthys stellatus,* the starry flounder (Wood et al. 1979). This is 3-4 times higher than the pressures we measured in the veins of the equally inactive *E. cirrhatus*. In more active teleost species venous pressures are higher again. Pressure in the common cardinal vein of the trout (*Salmo gairdneri*) has been measured as 1.9cm H_2O (Kiceniuk and Jones 1977) and pressures in the gill veins of the Lingcod (*Ophiodon elongatus*) were measured between 6.25 and 8.84 cm H_2O (Farrell and Smith 1981).

With extremely low central venous pressures (venous return) the filling rate of the hagfish heart must be slow, especially as hagfish lack a rigid, closed pericardium which would facilitate faster aspirational filling (*vis-a-fronte*) of the heart (Forster 1998). It is likely that hagfish rely on *vis-a-tergo* filling of the systemic heart (force from behind), which is dependent on venous return. With *vis-a-tergo* filling, the rate of filling depends on the pressure differential between the atrium and pressure in the sinus venosus (Farrell 1991).

A diastolic pressure of 0.2cm H_2O has been measured in the atrium of *M. glutinosa* (Satchell 1986a). As this is at the lower end of central venous pressures that we measured , it is feasible that if similarly low pressures exist in the atrium of *E. cirrhatus,* an adequate pressure differential would exist to support slow *vis-a-tergo* filling. We do not know what normal pressures are in the atrium of *E. cirrhatus,* however, as long as intra-atrial pressure remains lower than central venous pressure, an average of 0.39 cm H_2O in this case, then filling of the systemic heart would proceed. With filling of the heart depending on a relatively small pressure differential, the importance that physical activity may have on the filling of the heart is emphasised. Our work supports *vis-a-tergo* filling in the systemic heart of *E. cirrhatus* as the resting venous pressures seen in table 2.1 were typical, and sub-ambient pressures were not recorded in the posterior cardinal vein during the study.

The tip of the cannula, which we used to measure central venous pressure (PCV), was placed as close to the systemic heart as possible. Occasionally we recorded a small pressure pulse in the PCV which was out of phase with the pressure pulse recorded simultaneously in the DA, suggesting tip placement in the sinus venosus, the pacemaker of the systemic heart (Davie et al. 1987). Even though a downward stroke in pulse pressure was observed,

pressure still remained higher than ambient in these cases. Pressures recorded in the PCV were low but positive regardless of treatment, suggesting *vis-a-tergo* filling with no evidence of *vis-a-fronte* filling.

Central venous pressures as close to zero as we measured, imply low venous return which must influence cardiac output, especially as hagfish hearts conform to the Frank-Starling mechanism whereby decreased venous filling pressure would act to decrease stroke volume and cardiac output (Chapman et al. 1963; Forster 1989). Nevertheless, measurements of CO in hagfish have been shown to be comparable with the CO of many other types of fish of similar athletic ability (Forster 1998).

Heart Rate in *E. cirrhatus*

As a consequence of low central venous pressures, the heart rate of hagfish must be slow to allow time for adequate filling of the heart. Hagfish have some of the slowest heart rates recorded among vertebrates and *E. cirrhatus* is no different. We measured a mean resting heart rate of 24 beats per minute in 15 fish. The majority of teleosts maintain heart rates higher than this, in the range of 20-60 beats per min (Farrell 1984).

The structure of the hagfish heart appears to preclude it from operating at higher frequencies, as there is an absence of a coronary circulation, fast conducting tracts and transverse tubules that would facilitate the frequencies seen in teleosts (Helle and Lonning 1973; Satchell 1986a). Additionally the long conduction time of the action potential of the systemic heart is remarkable (Arlock 1975; Davie et al. 1987). The inability of the hagfish heart to generate high frequencies and power has been seen as a primitive trait. However, in view of the low pressure, moderate flow cardiovascular system of the hagfish, low frequency and power generation may be an advanced adaptive feature (Forster 1998). It is conceivable that this type of system with exceptionally low energy requirements may have evolved, driven by selection pressures, selecting for the advantages of energy conservation (Forster 1998).

Forster et al (Forster et al. 1992) report a mean heart rate of 27 beats per minute recorded *in vivo* in hypoxic *E. cirrhatus* at 17 $^{\circ}$ C. This is the highest mean systemic heart rate reported in the literature for any hagfish, reiterating the generally slow rates recorded in these animals.

The ability of hagfish to maintain a cardiac output similar to those of other equally inactive fish, must therefore, depend on a comparatively large stroke volume. Because of this, increases in heart rate without a concomitant increase in cardiac filling pressure are likely to compromise cardiac output by reducing the time available to fill the heart.

Volume Manipulation

With such a unique cardiovascular system, the study of the effects of volume manipulation on cardiovascular parameters of *E. cirrhatus* has given some exciting insights into cardiovascular control systems in this ancient vertebrate. Table 2.1 reports absolute val-

ues of the measured cardiovascular parameters early in volume manipulation (60 minutes) and Figure 2.4 shows the same cardiovascular parameters expressed as the percent change from resting values over a longer period post volume manipulation (150 minutes). Table 2.2 & 2.3 apply ANOVA and linear trend tests not only to the 150 minute data set but also to shortened data sets (60 and 90) minutes to help clarify time points after trends in cardiovascular parameters change.

At 60 minutes (Table 2.1) after the induction of volume manipulation, absolute values of DA, SIV and pulse pressure had all changed significantly matching the changes made to the external medium (although when calculated as a percent change from resting values the increase in DA with volume loading and the decrease in SIV with volume depletion are not significant, see figure 2.4a & 2.4c). This is in agreement with the findings of Rudy and Wagner (1970) that hagfish are permeable, and shows that intravascular dynamics are changing rapidly, in this case by 60 minutes.

The changes in pulse pressure are noteworthy (Table 2.1 and figure 2.4e) as alteration of this parameter can indicate changes in; the force of blood propulsion, the intravascular volume, the interstitial fluid load and/or changes in vascular tone and compliance in the arterial system (Olson 1998). The observed changes in pulse pressure support the hypothesis that intravascular dynamics have changed as a result of volume manipulation, although it is difficult to attribute the specific cause from this data. Increases in the recorded pulse pressure, would be expected with an increase in transmural pressure in the vessel (and vice versa for a decrease in transmural pressure). Increases in transmural pressure are to be expected with volume loading and are likely to indicate increased intravascular volume and/or interstitial fluid load, this demonstrates the rapid effects of volume manipulation.

Cardiovascular Events During Volume Loading

Cardiovascular parameters changed markedly during volume loading, however, by the 150 minute time point, intravascular pressure homeostasis been achieved (

Central venous pressure was the only parameter that did not respond in the expected manner with volume loading i.e. it did not show even a transient increase above resting pressures (2.4b). Intriguingly, there was a significant decrease in central venous pressure early in volume loading (table 2.1, figure 2.4b, and table 2.3) that preceded the compensation in pressure observed in other vessels. Restoration of PCV pressure towards resting levels occurred after the other parameters began to decline towards resting values.

DA pressure peaked at 40 minutes, after which, compensation was observed and pressure decreased to be similar to resting values by 100 minutes (figure 2.4a). For the remaining 50 minutes, DA pressure remained remarkably stable, approximating resting values. Dunnett's post test did not detect a significant change at any particular time point for this parameter (although significance was observed with the shorter data sets, table 2.3) which suggests good compensation of arterial pressure after an initial transient increase to just

over 110% of the average resting pressure. The qualitative changes in SIV pressure were similar to those observed in the DA, however as a proportion of resting values, pressure changed more (figure 2.4c). There was also a transient increase in heart rate although why this occurred is not clear. It seems likely that a pressure sensitive pacemaker mechanism exits in the hearts of hagfish as positive chronotropic effects have been observed with increased filling pressure/volume (Jensen 1961; Bloom et al. 1963; Chapman et al. 1963; Johnsson and Axelsson 1996; Johnsson et al. 1996). However the concomitant decrease in central venous pressure discounts this as an explanation for the transient increase in rate. It is possible that a humoral element may be mediating this response but as can be seen in Chapter Four, this does not correlate with a release of catecholamines and we did not assess any other humoral agents in blood samples.

It is clear that *E. cirrhatus* possesses compensatory systems to assist cardiovascular homeostasis when challenged with hypertension. The decrease in PCV pressure during loading is likely to be beneficial as reductions in cardiac filling generally reduce stroke volume and therefore cardiac output in fish (Farrell 1991). This may help to compensate the increased pressure that was observed in the arterial circulation early in volume loading. The potential influence of the proportionally large decrease in PCV pressure is great, given the incredibly small pressure differential between the sinus venosus and the atrium that determine cardiac filling under normal conditions (Satchell 1986a). With pressures as low as we observed in the PCV, it is likely that cardiac output would have been severely compromised. It is therefore not surprising that this parameter returned to resting values within a comparatively short time frame after the induction of volume loading (at 120 minutes figure 2.4b) as prolonged decreases could have deleterious effects.

That central venous pressure actually decreased during volume loading and did not simply maintain resting pressure suggests that this cardiovascular parameter may be being actively regulated in this animal during this treatment.

However, the possibility that this compensation could be mediated by a passive mechanism such as a venous shunt, can't be excluded (see discussion pertaining to the subcutaneous sinus earlier). The possible overflow of blood presumably under increased pressure does not adequately explain the disproportionate decrease in central venous pressure in comparison to increased pressures in other parts of the circulation. If the observed decrease in PCV pressure was passive, i.e. that the central circulation was open to the sinus system and this provided an overflow valve into this expansible compartment, then it might be expected that central venous pressure would not decrease, but maintain resting pressure (although a transient increase might be observed if there was resistance at the points of entry to the sinus). It has been suggested that increased physical activity in hagfishes might boost cardiovascular function by increasing the cardiac filling pressure (Axelsson et al. 1990; Forster 1998). If this is true then it may be possible that activity of the animal could force extra blood into the venous sinus system during volume loading, leaving the central circulation depleted which could account for the observed decrease in PCV pressure but this is unlikely for the following reasons:

1) Overflow of blood into the sinus system so as to functionally deplete the central circulation would appear to be maladaptive. This infers that after activity cardiovascular function would be compromised and this would not aid post exercise recovery. If central circulating volume was depleted in this manner, blood pressures would be expected to decrease after exercise (in the absence of an opposing compensatory mechanism such as homeostatic vasoconstriction of the central vasculature). Forster et al (1988) measured DA and ventral aortic (VA) blood pressures in *E. cirrhatus* at rest, during swimming and during recovery. During recovery these workers a report a higher pressure in the DA than while swimming and at rest and no difference between resting and recovery values for VA pressure. This is inconsistent with a loss of extra blood into the sinus system as a result of activity, however changes in vascular compliance and tone were not measured during this work.

2) A significant depletion of the central vasculature would likely be correlated with decreases in pressure in the parts of the circulation other than just the PCV (again in the absence of compensatory vasomotion) and this was not observed to occur in this study. Although figure 2.4 shows other cardiovascular parameters to be restored to approximate resting values late in volume loading, changes in SIV and DA pressures lag the decrease seen in central venous pressure.

3) Some animals showed decreased central venous pressures with volume loading and did not display any physical activity at all.

4) Additionally there is no evidence that blood might be actively transferred into the sinus system and it is hard to imagine how such a mechanism would work (Forster 1997).

As volume manipulation was achieved by diluting the 100% seawater that hagfish were normally maintained in, it is possible that a change in the ionic composition of the blood and interstitial fluid could have a direct effect on vascular smooth muscle. Although iso-osmotic, hagfish blood is not completely iso-ionic with sea water, plasma concentrations of some divalent cat ions are regulated (Robertson 1974; Fange 1998). Plasma concentrations of calcium, potassium, magnesium and sulphate are significantly lower than the concentrations found in sea water (Morris 1965). This situation is common to many marine invertebrates but is not seen in marine vertebrates, where high concentrations of organic substances such as urea or TMAO (trimethyl amine oxide) are used to achieve osmotic equilibrium of the plasma with seawater. McFarland and Munz (1965) reported that during dilution stress (volume loading) blood sodium and chloride in the Pacific hagfish declined in proportion to their decrease in the medium, but magnesium and calcium remained at initial concentrations. In work on the hypoxic vasoconstriction of cyclostome aortas Russell et al. (2001) reported that extracellular calcium accounted for 38% of the hypoxic vasoconstriction observed in *E. cirrhatus* DAs. These workers also showed that removal of extracellular sodium caused a constriction of the DA. What affect the 10% decrease in the osmolarity of the external medium might have on the vasculature of *E. cirrhatus* is not known. However, this is unlikely to have affected the vasculature in such a way to cause the observed decrease in PCV pressure, as central venous pressure was

restored to resting values after 120 minutes with no corresponding change in the ionic components of the external medium.

For the aforementioned reasons it seems that the compensatory mechanisms that allowed restoration of resting values could include active regulation of the central venous vasculature in this fish. It is possible that an increase in compliance or active vasodilatation of the central veins could act to reduce transmural pressure. As we have shown *in vitro* (Chapter Three and other unpublished work), *E. cirrhatus* veins respond vasoactively to a variety of neural and humoral elements and this could provide a mechanism for cardiovascular control *in vivo*.

In higher fish there is growing evidence for the active control of venous tone *in vivo*, (Conklin et al. 1997; Olson et al. 1997; Zhang et al. 1998). Zhang et al (1998) provided the first evidence for tonic regulation of vascular capacitance in any fish, when they showed that the sympathetic nervous system in rainbow trout is an important effector of venous tone and compliance.

With an apparent lack of innervation of the vasculature (Nilsson 1983), hagfish would most likely have to rely on humoral agents to exert regulatory effects. This is consistent with a longer time (∼100minutes) to restore homeostasis of blood pressures such as in this study.

Cardiovascular Events During Volume Depletion:

In contrast to the situation during volume loading, *E. cirrhatus* displayed a general lack of blood pressure homeostasis with volume depletion. DA pressure decreased significantly to between 80% and 90% of resting pressure and did not recover (figure 2.2a & 2.5b). Similarly, PCV pressures decreased with no compensatory effect comparable to that seen during loading (figure 2.4b & 2.5b). SIV was the only vessel in which there may have been some compensation as there was a significant positive linear trend in the 150 minute data set for this variable (table 2.2). This slight compensation is interesting as this vessel was shown to give the most potent responses of the vessels tested in *in vitro* myography (Chapter Three).

Heart rate did not change from resting values with volume depletion. As mentioned previously there are physical limitations on heart rate in hagfishes and studies involving the application of stimulatory factors found a maximum increase of 8 beats per minute (Forster et al. 1988; Axelsson et al. 1990). That hagfish were not able to increase heart rate above resting during depletion, combined with the measured decrease in filling pressures, supports the theory that cardiac output would be compromised during depletion. During volume depletion there was a general loss of pressure homeostasis indicating the absence of any other effective regulating mechanism i.e. vasoconstriction.

If hagfish were able to actively alter their venous tone and compliance and had reduced the capacitance of the venous vasculature it is possible we may have seen a maintenance of central venous pressures which would help to support cardiac output.

Because the venous side of the circulation of vertebrates contains the majority of the blood volume (Rothe 1986), any change in venous tone and or compliance may have comparatively large impacts on cardiovascular function by altering the "stressed blood volume" (Olson et al. 1997; Olson 1998; Zhang et al. 1998). Olson (1998) explains the concept of stressed and unstressed blood volume. "The unstressed blood volume (USBV) is, by definition, the volume of blood that would remain in the vasculature if the vessels were open to the atmosphere (MCFP=0) and the vessels passively collapsed. This is dead space and is hemodynamicaly inert, i.e. it does not contribute to venous return or cardiac output". "The stressed blood volume (SBV) distends the vasculature and, in combination with the vessels (mainly veins) establishes the mean circulatory filling pressure (MCFP)- the overall pressure in the static system which is driving venous return" (Olson 1998). Hagfish have a low pressure, moderate flow circulatory system. It is possible that there is a requirement to maintain lower pressures as the heart does not generate a large power output and cannot work efficiently against increased afterload.

Cardiovascular Events After 24 Hours of Volume Manipulation:

Cardiovascular events were qualitatively similar at 24 hours of volume manipulation as they were in the short term, immediately after induction. The animals had maintained the ability to apparently regulate pressures better during volume loading than volume depletion, to such an extent that DA pressure was actually lower than the resting pressure at 24 hours of volume loading (table 2.4). The mechanisms involved in this compensation in the longer term may be different to those evoked in the short term, immediately after induction. It is possible that in the short term more rapidly acting control systems may be switched on (i.e. plasma catecholamines and natriuretic peptides), that may act to alter vascular capacitance and therefore serve the acute need to minimise the effects of volume loading. In the longer term i.e. 24 hours it is more likely other control systems may be activated such as an upregulation in the urine production. Having said this, hagfish can only produce urine that is isotonic to sea water, so during depletion they would not be able to regulate the extra ionic load (Riegel 1998). Additionally urine production rate is likely to be reduced by the concomitant decrease in blood pressure. As a general conclusion it was apparent that *E. cirrhatus* did not tolerate volume depletion, little if any compensation in cardiovascular parameters was observed when animals were exposed to this treatment. Conversely on volume loading animals were able to restore cardiovascular homeostasis in the short term and compensatory mechanisms were still apparent at 24 hours after the induction of this treatment.

Chapter 3

Catecholamine Myography

3.1 Introduction
Vasoactivity in hagfish- Veins the Forgotten Vessels

There exists in the literature an emphasis on arteries and the role they play in cardiovascular functioning. Great importance has been placed on the role of active vasomotion of arteries in the regulation of blood pressure and flow. This is because arteries and arterioles, which are well endowed with smooth muscle and are well innervated, are able to alter their calibre and change their resistance to blood flow. Studies of the mechanisms controlling alterations in arterial tone and compliance point to various endocrine and autonomic nervous system products as effectors of this change.

In contrast to the dynamic portrayal of arteries, veins have been largely seen as passive conduits, particularly in fishes, because they contain little smooth muscle, are poorly innervated and are of a larger calibre than arteries. Active vasomotion of fish veins as an effector of cardiovascular control has, in the past, been seen as unlikely especially as fish live in an essentially gravity free environment (Satchell, 1991).

The suggestion that veins are not important effectors of fish cardiovascular control has been challenged by the work of a number of fish physiologists. (Wood and Shelton, 1980) found that adrenaline injection into the caudal veins of rainbow trout caused an immediate rise in venous pressure, thought to be due to venoconstriction. Bluefish subjected to head up tilting out of water actively prevented venous pooling of blood(Ogilvy and Dubois, 1982). Olson and colleagues have shown that the veins of salmonids react to a number of agents that also affect fish arteries including catecholamines, natriuretic peptides and endothelins(Conklin and Olson, 1993; Olson et al., 1997). It is now clear that trout can actively regulate mean circulatory filling pressure and changes in central venous pressures have a large potential influence on cardiac output (Olson, 1998).

The work presented in Chapter 2 showed that *Eptatretus cirrhatus* was capable of responding to acute volume change. This research demonstrated that these animals were able to regulate cardiovascular parameters better during volume loading than depletion. As a natural progression from this work, the potential vascular component of this regulation was examined by means of myography. This *in vitro* protocol allows investigation of mechanisms that alter vessel tension and therefore vessel calibre. Pharmacological studies employing both endogenous and exogenous catecholamines were performed. The results of pharmacological studies demonstrate that hagfish vessels respond vasoactively to a range of agents, indicating that control of venous tension has been possible from the earliest stages of chordate evolution.

This work provides further evidence that regulation of the hagfishes venous system has the potential to act as the effector in the pressure regulation we observed *in vivo* in the volume manipulation experiments presented in the previous chapter. It also suggests a possible role for the venous system in influencing cardiac output.

3.2 Methods

Vessel reactivity to the catecholamines; adrenaline (AD), noradrenaline (NA), phenylephrine (PHE) and isoprenaline (ISO) was tested on three veins; the left anterior cardinal vein (ACV), the left posterior cardinal vein (PCV) and the supraintestinal vein (SIV) of *E. cirrhatus*. The endogenous catecholamines possess both α and β stimulatory activity, with differing affinities (Ahlquist 1948, Ahlquist 1967). The exogenous compounds at low concentrations are pure agonists; Phe stimulates α_1- whilst Iso stimulates β-adrenoceptors (Wollmuth et al. 1988; Moon and Mommsen 1990; Crocker et al. 2000; Griffith 2003; Kozaka et al. 2003).

New Zealand hagfish were collected off Motunau beach or Akaroa harbour, New Zealand and were transferred to the University of Canterbury in Christchurch where they were held in aquaria containing circulating sea water (12-14 $^\circ$C). They were held at least one week prior to experimentation, and were not fed during this period.

Hagfish were anesthetized in seawater containing benzocaine (0.4g.L^{-1}) and MS222 (ethyl-*p*-aminobenzoate and 3-aminobenzoic acid ethyl ester metanesulfonate, 0.4g.L^{-1}), and vessel segments were dissected out, rinsed with a modified hagfish HEPES buffered saline (HHBS) and stored in fresh HHBS at 4 $^\circ$C for a maximum of two days until use. Preliminary experimentation demonstrated no reduction in vessel responsiveness over this period.

Vessels were collected from the same region of the vasculature to minimize variations in vessel composition. The preparation was handled carefully so as to avoid stretching. Veins were cut transaxially into 3-4 mm long rings (vessels could not be normalised for weight as it was not always possible to dissect them cleanly without risking damage, so great care was taken to ensure vessels were cut to accurate lengths). Rings were hung on 280 μm stainless-steel hooks and suspended in 20 ml, water-jacketed (12-14 $^\circ$C) smooth muscle chambers (Olson and Meisheri 1989) containing HHBS. Drugs were administered directly to the saline bathing the vessels. Vessel tensions were measured with Ugo Basile (Comerio, Italia) isometric force transducers (model 7004) and the signals were amplified with Gould (Valley View, Ohio) transducer preamplifiers (model 13461550). Signals were displayed and digitized on a Yokogawa LR4100E recorder (Yokogawa Electric Corporation, Tokyo, Japan.

Optimal resting tension for the veins was determined in preliminary experiments by measuring the magnitude of acetylcholine (10^{-4} mol.L^{-1}) contractions over a range of resting tensions from 200 to 800mg. A resting tension of 300-400mg was subsequently applied to all experimental vessels for at least one hour prior to experimentation. Note that KCl was not used to depolarise vessels, as it fails to elicit maximal responses, which might be explained by hagfish plasma having higher potassium concentrations than is found in other chordates (Robertson 1976).

Following experimentation, vessels were exposed to the acetylcholine analogue carbachol (CBC 10^{-5}-10^{-4}mol.L^{-1}) to test for viability. Myograph traces from vessels that

failed to respond to CBC were excluded from analysis. Sotalol a non-selective β_1 and β_2 antagonist (Axelsson et al. 1987; Borchard 1998) was applied to vessels in some preliminary experiments to confirm β activity.

The composition of hagfish HEPES buffered saline was as follows (in g.L $^{-1}$: 27.70 NaCl, 0.60 KCl, 0.75 $CaCl_2$.2 H_2O, 0.75 $MgSO_4$.7H_2O, 0.72 HEPES acid form, 1.82 HEPES sodium salt, 1.00 glucose, pH 7.8). Carbamoylcholine chloride (carbachol), Adrenaline, Noradrenaline, Phenylephrine hydrochloride and Isoprenaline hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Sotalol hydrochloride was a gift from Michael Axelsson.

Cumulative concentration response curves were constructed to determine vessel sensitivity to catecholaminergic agonists and bar graphs of absolute changes in tension allowed assessment of relative potency. Sensitivity was expressed as the log EC_{50}, the negative logarithm of the agonist concentration that elicits the half maximal response.

Concentration response curve data were normalized by conversion to the percent of maximal response. As curves were expressed in this way, the base and tops of the curves were constrained between 0 and 100% (constrictor responses = 0 to 100%, dilatation responses= 0 to -100%).

Graphpad software was used to generate graphs and perform statistical analysis. Repeated measures ANOVA with Dunnett's post test were employed to detect significance unless otherwise stated. The limit of significance was $P < 0.05$.

3.3 Results

3.3.1 Preliminary Myography Experiments Using AD and NA

Response of *E. cirrhatus* ACVs to Endogenous Catecholamines

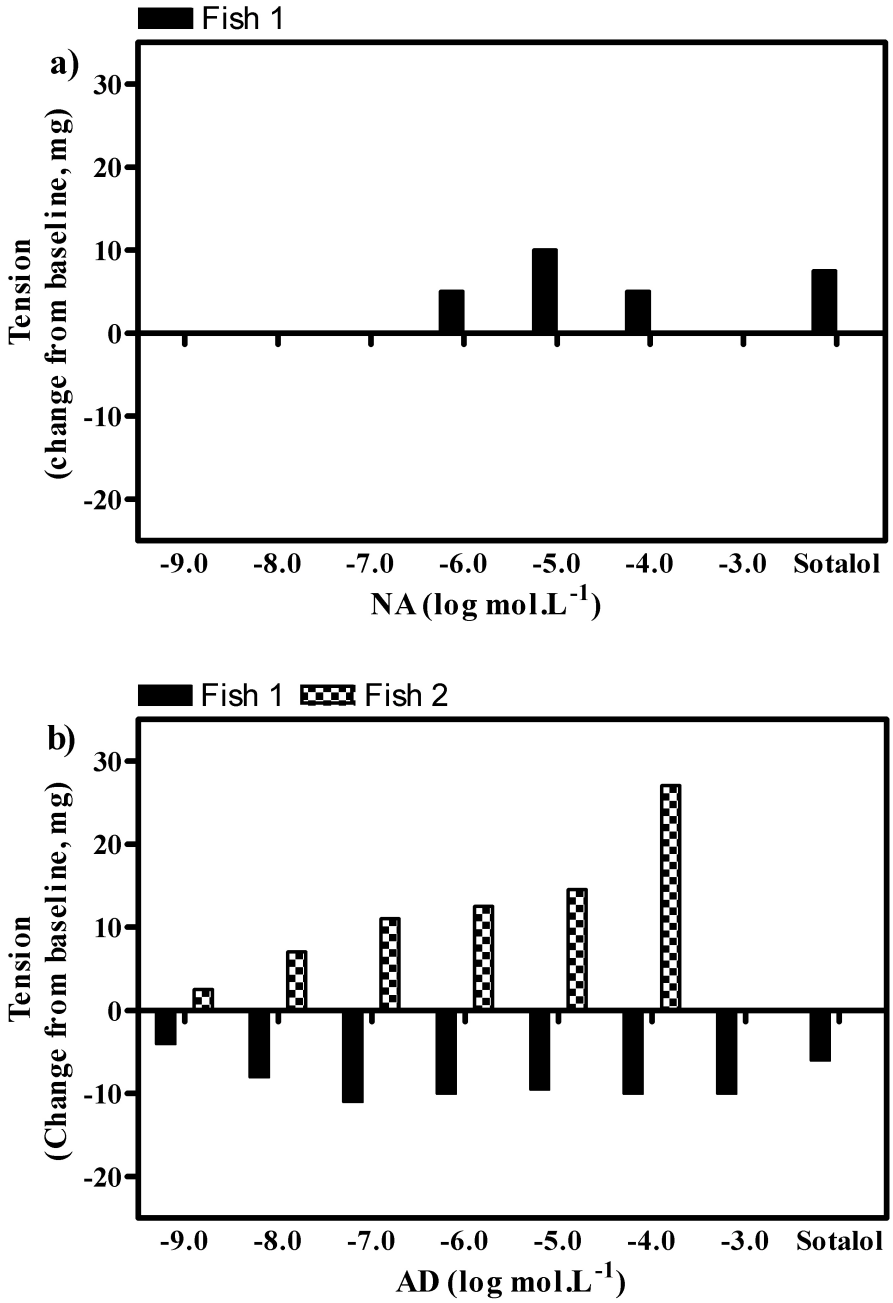


Figure 3.1: Changes in tension of hagfish ACVs in response to increasing concentrations of noradrenaline (NA) and adrenaline (AD). Veins received the full range of concentrations displayed on the X axis, with the exception of Fish 2 which did not receive the final concentration of 1x10^{-3} mol.L^{-1}. Sotalol (β antagonist) was administered at a concentration of 5x10^{-3} mol.L^{-1}after the final catecholamine dose for vessels collected from Fish 1.

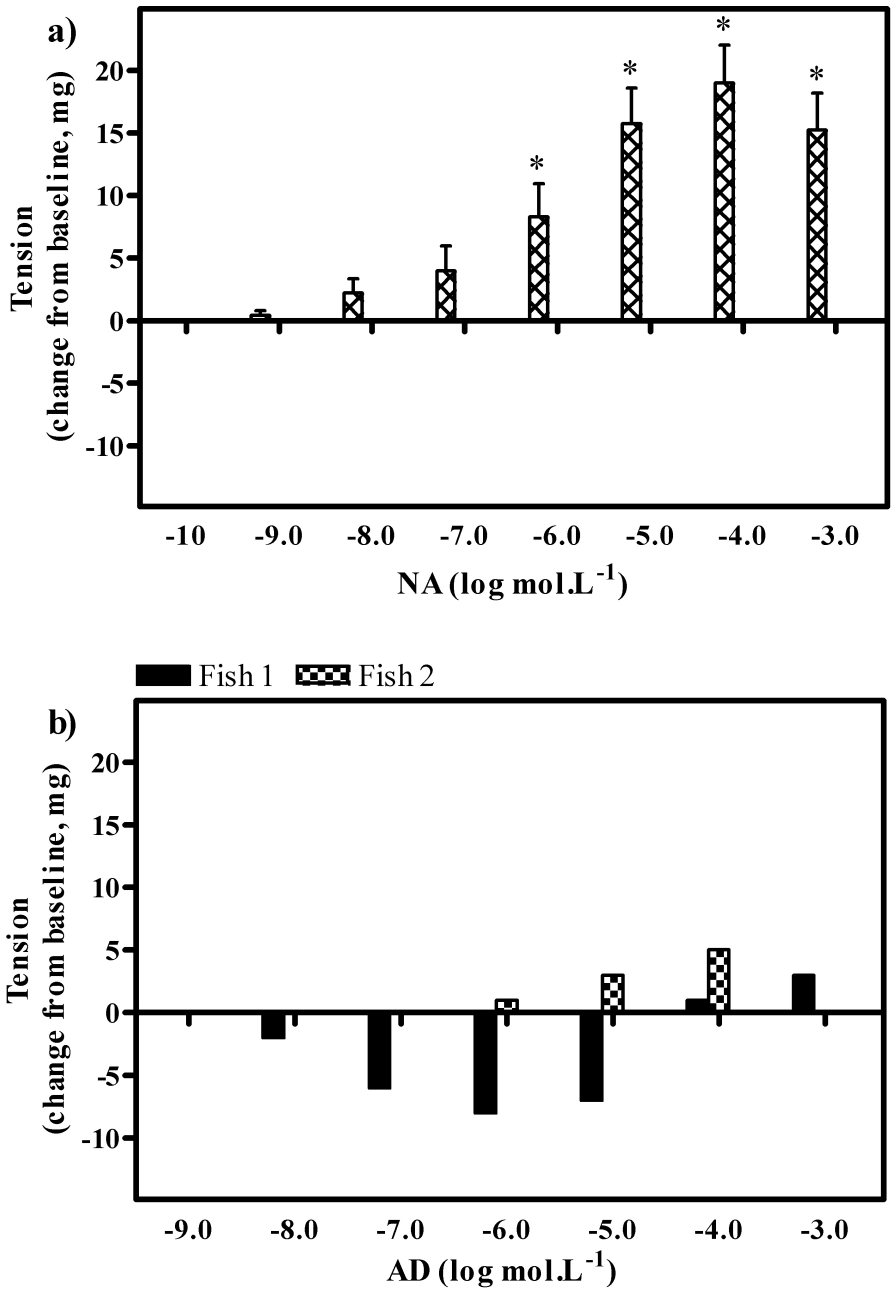
Of three ACVs tested (collected from separate animals) only one vein responded to NA (figure 3.1a).NA elicited increases in vessel tension past a comparatively high threshold dose of $1x10^{-6}$ mol.L^{-1} (1000nM). This constrictor response was not incrementally concentration dependent as tension decreased again at higher doses returning to baseline tension at $1x10^{-3}$ mol-L^{-1}. The subsequent administration of sotalol caused an increase above baseline in the presence of $1x10^{-3}$ mol.L^{-1} NA.

Exposure to AD produced concentration dependent tension decreases in the vessel collected from Fish 1. β-blockade with sotalol reduced the vasodilatory effect of AD but did not return it to baseline values.

Fish 2 displayed the opposite response, with dose dependent increases in tension peaking at the maximum concentration administered of $1x10^{-4}$ mol.L^{-1}.

Of the three veins that received AD, one responded with vasodilatation (Fish 1 figure 3.1b) and two displayed vasoconstriction (Fish 2 fig 3.1b). The response from the third ACV was a weak vasoconstriction <5mg tension increase) and is not shown here.

Response of *E. cirrhatus* PCVs to Endogenous Catecholamines



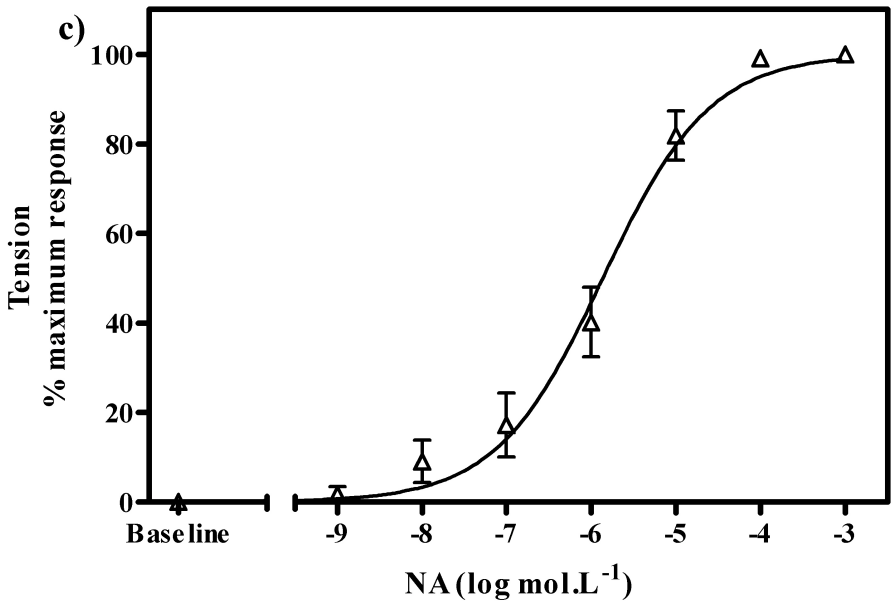
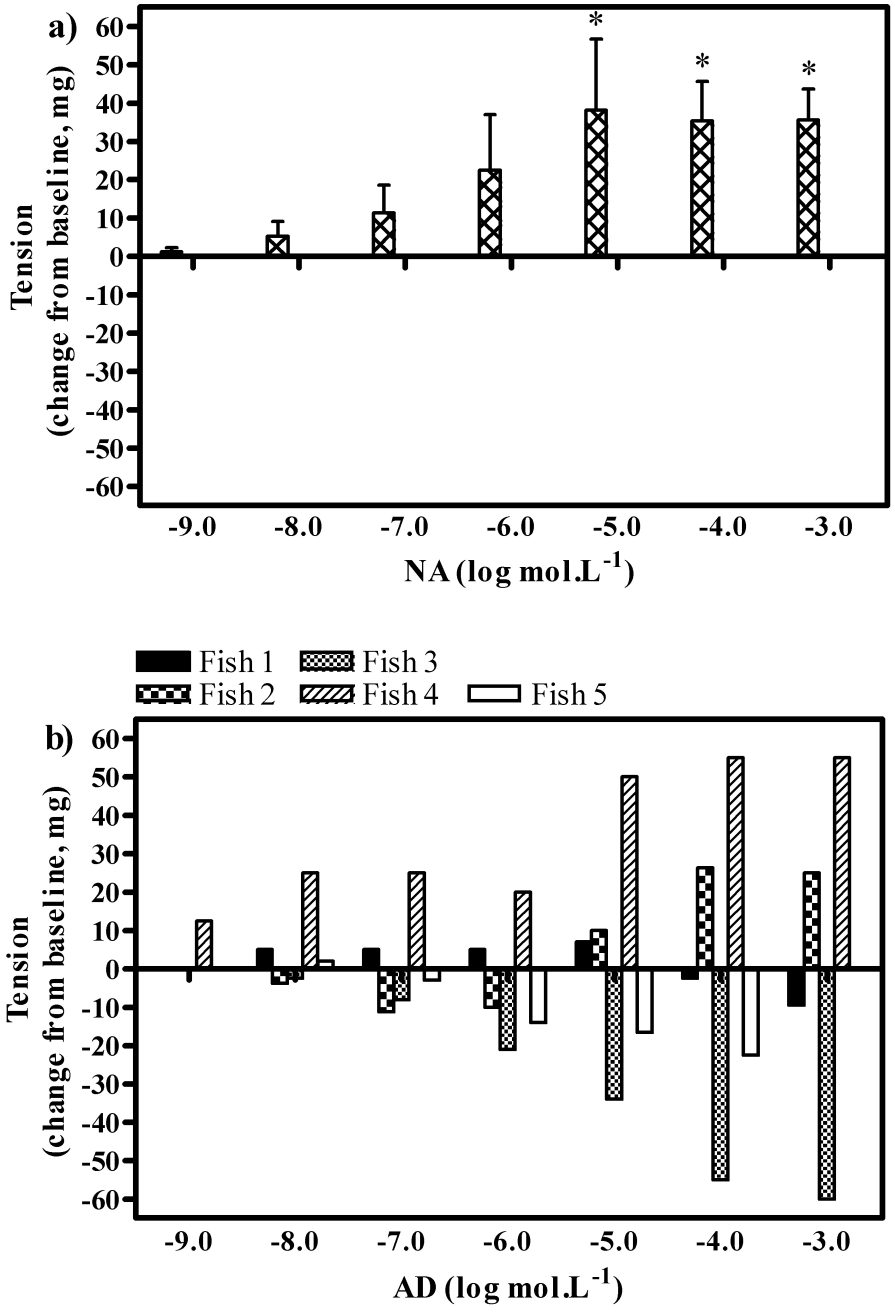


Figure 3.2: Changes in vessel tension of PCVs to NA and AD. **a)** Average change in absolute tension of *Eptatretus* PCVs with NA (n=5 except at 10^{-3} log mol.L^{-1} where n=4) * indicates significance (P<0.05). **b)** Response of PCVs from two fish to AD. Note Fish 1 and 2 are different to those employed in figure 3.1. **c)** NA data (2a) presented as a cumulative concentration response curve. Log EC_{50}= -5.86 ±0.09 (mean ±S.E.M.). n = 5.

NA caused concentration dependent increases in PCV tension. This became significant at the relatively high concentration of 1x10^{-6} mol.L^{-1}. The log EC_{50} was also high as can be seen from its value when converted to nanomoles per liter, 1369nM.L^{-1}. NA mediated venoconstriction peaked with an average increase of 19 ±3mg at 1x10^{-4} mol.L^{-1}. Of five separate PCVs tested with NA, none responded with a decrease below baseline tension.

As with ACVs, AD produced weak contradictory responses in the two PCVs tested. It should be noted that both vessels were capable of much stronger responses as they both reacted strongly to carbachol (CBC). The PCV from Fish 1 displayed a biphasic response with early depressor activity followed by weaker pressor activity at the highest doses whilst the vessel from Fish 2 showed only weak pressor activity above 1x10^{-6} mol.L^{-1}.

Response of *E. cirrhatus* SIVs to Endogenous Catecholamines



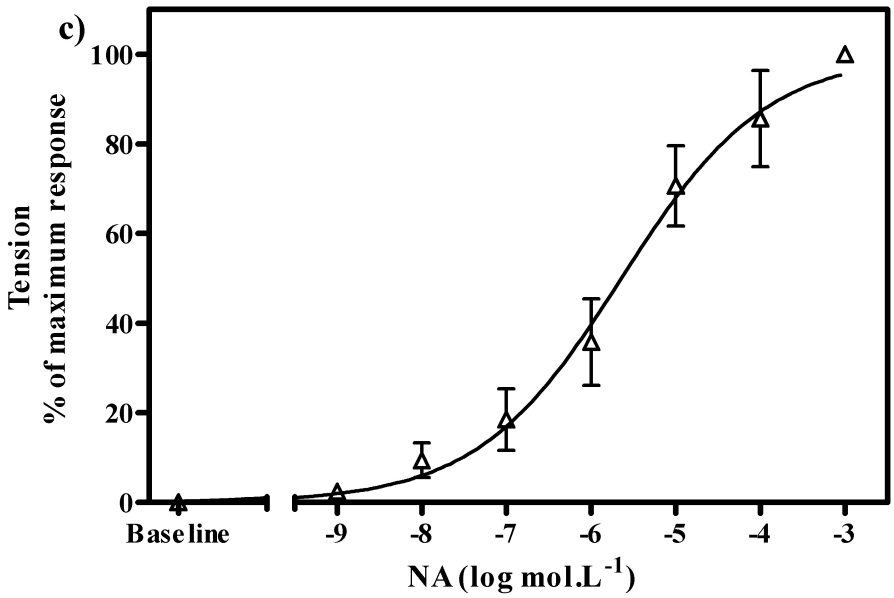


Figure 3.3: Changes in tension of SIVs to NA and AD. **a)** Average change in absolute tension of SIVs with NA (n=5 excluding 10^{-3} mol.L^{-1} where n=4) * indicates significance (P<0.05). **b)** Response of SIVs of five fish to AD. **c)** NA data (3a) presented as a cumulative concentration response curve. Log EC_{50}= -5.64 $\pm$ 0.14 (mean $\pm$S.E.M.).

NA again evoked dose dependent increases in vessel tension, peaking with an average increase of 38.2 $\pm$18.5mg (S.E.M) at 10^{-5} mol.L^{-1}. Although responses to NA were generally stronger in SIVs than the other two vessels tested, they were also more variable. Sensitivity to NA was relatively low, evidenced by the log EC_{50} concentration (equivalent to 2267nM.L^{-1}). Again none of the vessels tested with NA responded with a decrease below baseline tension.

AD produced an entire gamut of venomotion in the five *Eptatretus* SIVs tested. Responses observed include; dose dependent vasodilation, dose dependent vasoconstriction and biphasic responses including pressor followed by depressor and depressor followed by pressor responses at high doses. On the whole, responses to AD were more potent in SIVs than in the other veins with some veins exhibiting tension changes in excess of 50mg.

Exposure of hagfish veins to the endogenous catecholamines NA and particularly AD produced definite but complex and varying responses. Due to this, limited numbers of preliminary experiments were performed using endogenous catecholamines in favor of collecting full data sets employing α- or β-adrenergic agonists (see following section).

3.3.2 ISO and PHE Myography

Response of *E. cirrhatus* ACVs to Exogenous Catecholamines

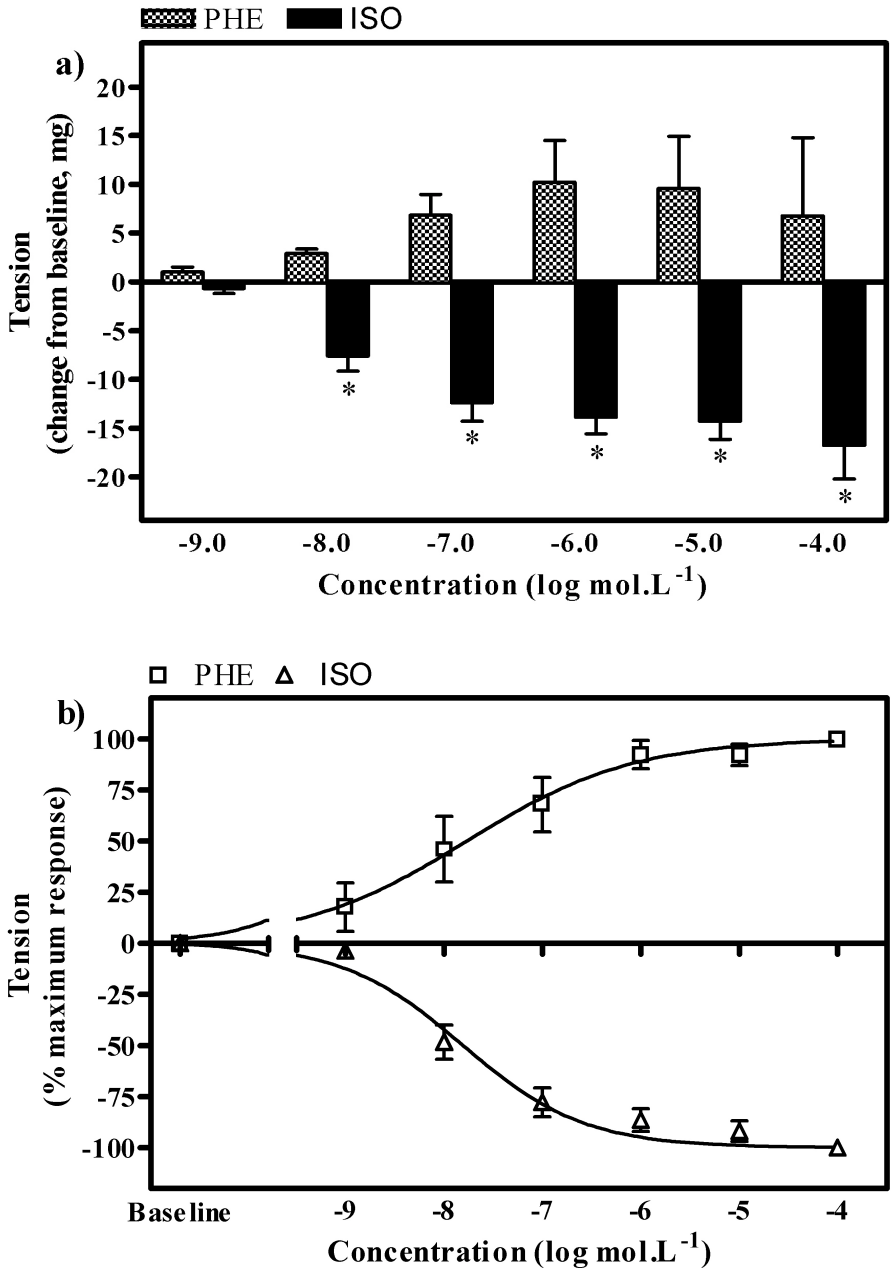


Figure 3.4: Changes in tension of hagfish ACVs to phenylephrine (PHE) and isoprenaline (ISO) **a)** Average changes in absolute tension of ACVs with PHE and ISO (n=6 & n=5-n=4 at the highest dose, respectively) * indicates significance ($P<0.05$). **b)** Data (4a) presented as cumulative concentration response curves. PHE log EC_{50}= -7.77 ±0.23 ISO log EC_{50}= -7.81 ±0.11 (mean ±S.E.M.).

PHE (α agonist) and ISO (β agonist) caused weak insignificant increases and moderate decreases in ACV tension respectively. Only the vasodilatation was significantly different to baseline. The vasodilation was more pronounced than seen with the endogenous catecholamine AD, although the constrictor response to AD was stronger than that elicited by PHE. There was greater variation in the constrictor response (PHE) than the dilatation response (ISO), especially at the strongest concentrations. The concentration dependent depressor effect of ISO became significant at a low concentration ($1x10^{-8}$ $mol.L^{-1}$ or $10nM.L^{-1}$) and attained the greatest change in tension (with a decrease of 16.75 $\pm$0.50mg) at the highest concentration, $1x10^{-4}$ $molL^{-1}$. Although ISO produced the most potent response, there was no difference in the sensitivity of ACVs to either exogenous agonist. ACVs were sensitive to both agents with log EC_{50}'s that equated to $17nM.L^{-1}$ for PHE and $16nM.L^{-1}$ for ISO.

Response of *E. cirrhatus* PCVs to Exogenous Catecholamines

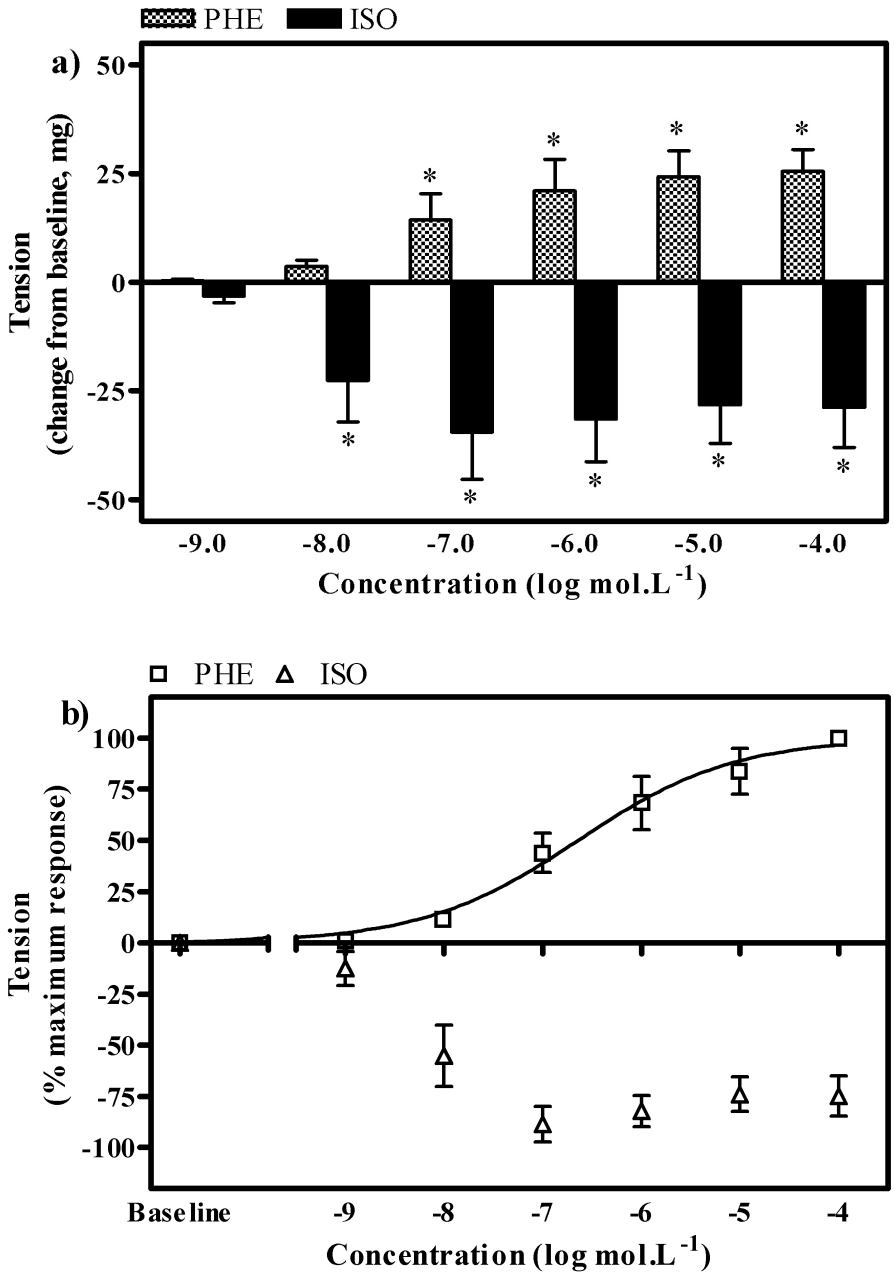


Figure 3.5: Changes in tension of PCVs to PHE and ISO presented as **a)** Average change in absolute tension of PCVs (PHE n=6 and ISO n=7). And **b)** as a cumulative concentration response curve. * Indicates significance ($P<0.05$). PHE log EC_{50}= -6.64 ±0.16 (mean ±S.E.M). A regression line could not be fitted to the ISO data, but the EC_{50} can be seen to be close to 1 x 10^{-8}mol.L^{-1}. All error bars are ±S.E.M.

In PCVs both the pressor effect of PHE and the depressor effect of ISO were significant in terms of potency. This venomotion was stronger than that observed in the ACVs. Venoconstriction peaked with an increase of 25.5 ±5.04mg at 1x10^{-4} mol.L^{-1}, whilst the vasodilatation reached its maximum earlier, with a decrease of 34.50 ±10.85mg at 1x10^{-7} mol.L^{-1}. A cumulative concentration response curve was fitted to the PHE data, which indicated sensitivity to this drug was moderate with an EC_{50} equivalent to 227nM.L^{-1} (see descriptive statistics above). Because the response to ISO peaked early in the series and diminished after its maximum, a curve could not be resolved, even when data were truncated after the maximum (100%). It is obvious from the shape of the graph (5b) that the PCVs tested were more sensitive to ISO than PHE.

Response of *E. cirrhatus* SIVs to Exogenous Catecholamines

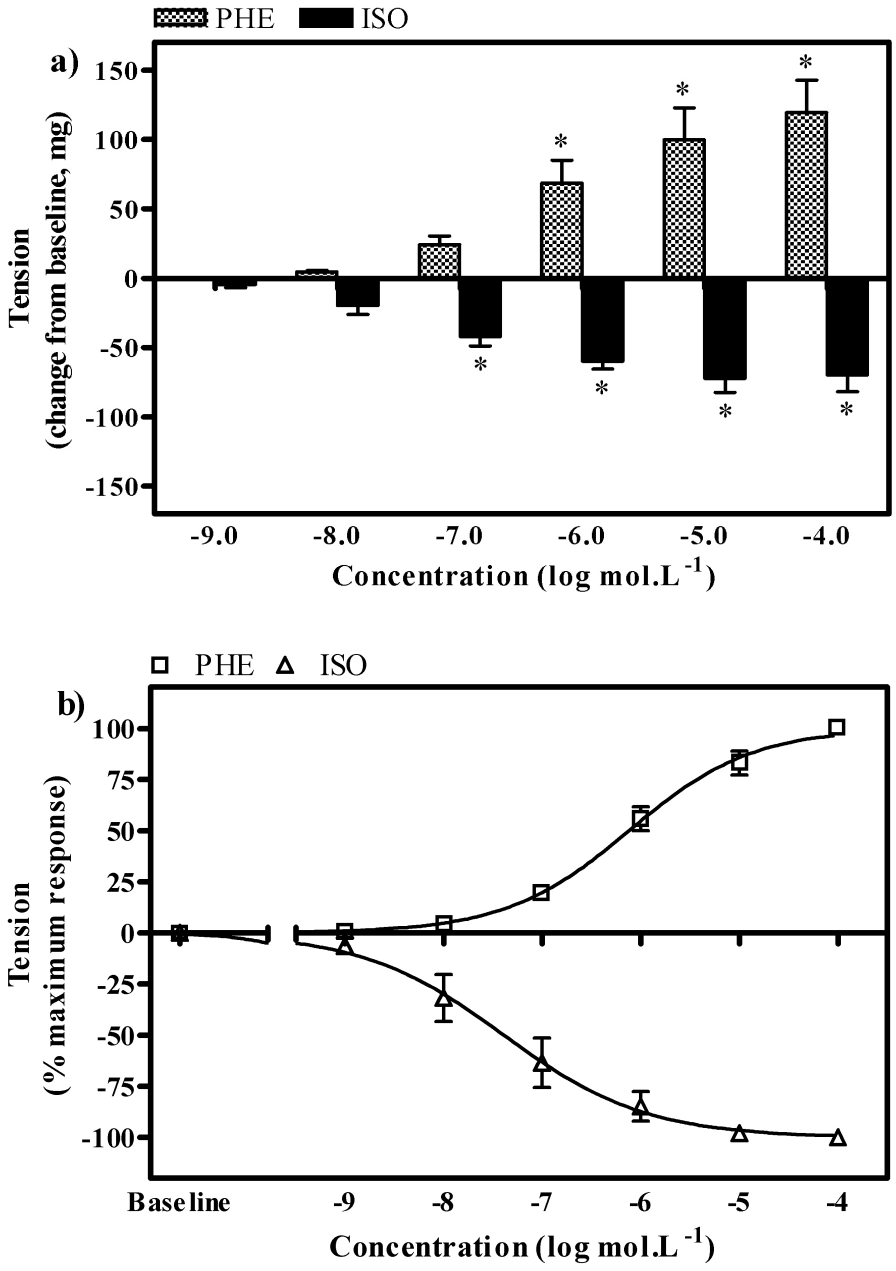


Figure 3.6: Changes in tension of SIVs to PHE and ISO presented as **a)** average change in absolute tension of SIVs with PHE and ISO (n=6) * indicates significance ($P<0.05$). **b)** Data (6a) presented as cumulative concentration response curves. PHE log EC_{50}= -6.12 ±0.06 ISO log EC_{50}= -7.39 ±0.14. (mean ±S.E.M.). The log EC_{50}'swere statistically different ($p<0.0001$)

PHE and ISO again caused significant increases and decreases in vessel tension respectively. In terms of absolute change, the greatest potencies (both vasoconstriction and vasodilation) were observed in the SIVs. PHE at $1x10^{-4}$ mol.L^{-1}induced the average maximum increase of 119 $\pm$23.25 mg. ISO at $1x10^{-5}$ mol-L^{-1} elicited the average maximum decrease of 72.17 $\pm$10.27 mg. At high concentrations PHE appeared to be the most potent of the agonists, however this was not true at lower, more physiological levels, with little difference between the two (possibly a slight dominance by ISO). Cumulative concentration curves were fitted to both data sets (descriptive statistics above). Although the maximum absolute change in tension was greater for PHE in this vein, there was a significantly greater sensitivity for ISO with an EC_{50} equivalent to 41nM.L^{-1} whereas the same parameter for PHE was 759nM.L^{-1}.

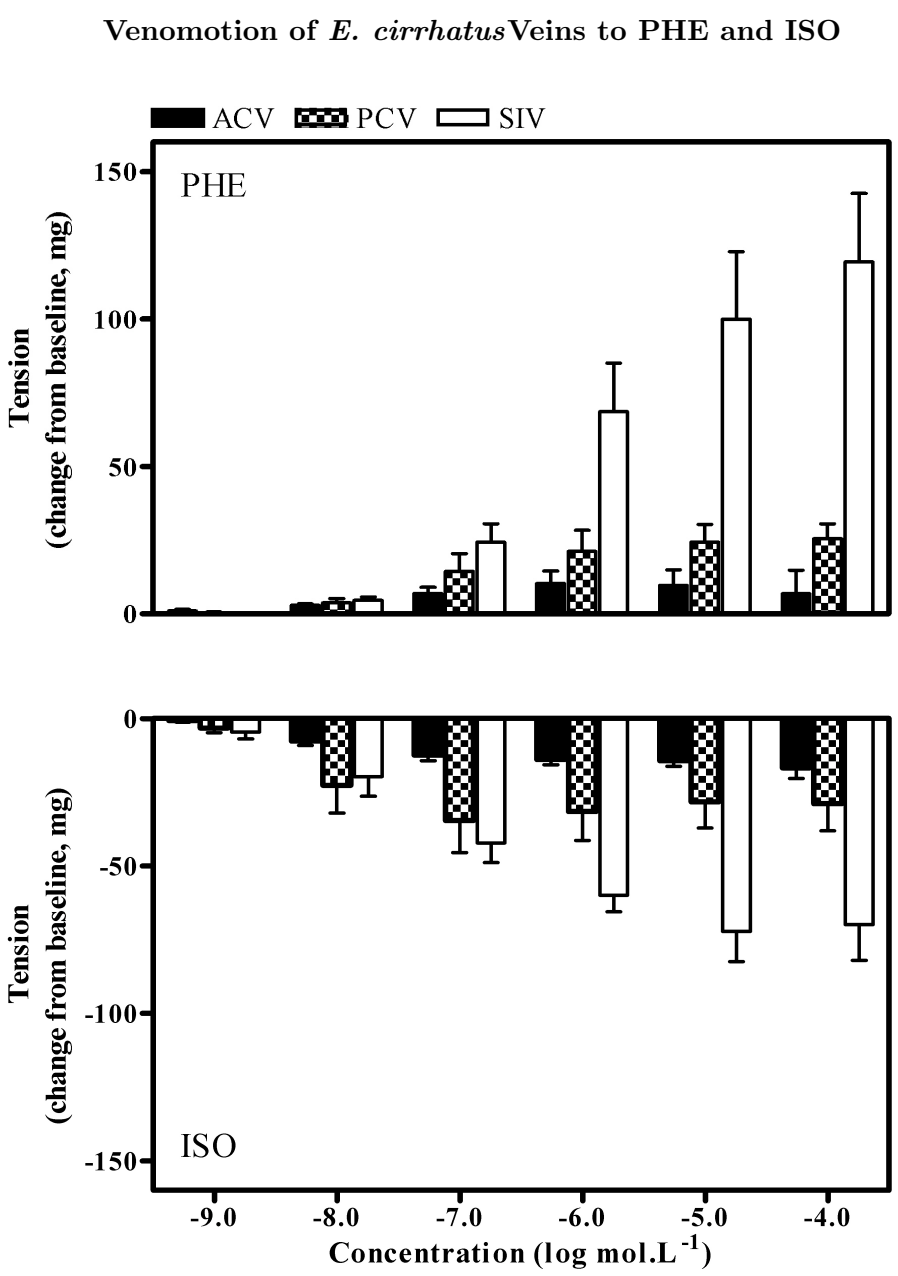


Figure 3.7: Changes in average absolute tension of ACVs, PCVs and SIVs to PHE and ISO. Data from figure 3.4a, 3.5a and 3.6a presented to show comparative differences in the responses of the three types of veins.

Responses to endogenous catecholamines were not strong when compared to responses elicited by the pure agonists. Overall SIVs displayed the greatest reactivity followed by the PCVs and then ACVs.

The response of the SIVs to PHE, was significantly greater than that of either the ACVs or PCVs (ANOVA, with Tukey's multiple comparison test, $P< 0.01$) and the response of the SIVs to ISO was significantly greater than that of the ACV ($P> 0.05$) but not the PCV.

When veins were tested for differences in the magnitude of response between the two drugs at each concentration, only the response at $1x10^{-7}mol.L^{-1}$ for ACVs was significant (Unpaired T test with Welch's correction $P<0.05$), with the vessel showing a significantly greater dilatation. The responses of all three veins approached significance at $1x10^{-9}$, $1x10^{-8}$ and $1x10^{-7}$mol.L-1 with a bias towards ISO mediated vasodilatation. Although proportionally greater variation within the data sets at low concentrations rendered the differences in magnitude insignificant, this does not mean that these differences are biologically unimportant.

3.4 Discussion

3.4.1 Catecholamines and Fish

In work on fish, much attention has been directed towards the vasomotion of the arterial system, with comparatively little on the venous system (Capra and Satchell 1974; Mustafa and Agnisola 1998; Zhang et al. 1998; Evans and Harrie 2001; Olson et al. 2001). I have begun to address a gap in the literature by surveying the responses of hagfish veins to adrenergic stimulation. This work has shown that the veins tested have the capacity to respond to adrenergic stimulation *in vitro* . This suggests that these veins are more than mere passive conduits for the return of blood to the heart and may in fact have the capacity to influence the venous return and hence cardiac function

Catecholamines are released into the circulation of fishes in response to physiological stressors which require modulation of cardiorespiratory function and/or mobilization of energy reserves (Randall and Perry 1992; Perry and Bernier 1999). One of the primary roles of circulating catecholamines in teleost fish is to maintain blood oxygen transport under conditions which depress blood oxygen content (Perry et al. 2005). During such challenges, in addition to their other actions, catecholamines act on the vasculature, altering tone and compliance to ensure efficient gas exchange and appropriate perfusion of tissues (Wood and Shelton 1980; Nilsson 1994; Sundin et al. 1994). In higher vertebrates including teleosts, catecholamines may originate from both chromaffin cells and sympathetic neurons, however it does not appear that sympathetic overflow from adrenergic nerve terminals, significantly contributes to elevations in plasma catecholamines in fish (Perry et al. 1991). In these higher vertebrates the sympathetic nervous system is an important (although not exclusive) mediator for the release of catecholamines from chromaffin tissue. As hagfish appear to lack much of the sympathetic innervation present in higher animals, humoral pathways must perform this function (Augustinsson et al. 1956; Jensen 1961; Jensen 1965; Nilsson 1983; Reid et al. 1998). There is evidence suggesting that catecholamines have a tonic role in controlling both cardiac function and branchial vasculature in hagfishes (Axelsson et al. 1990; Forster et al. 1991; Sundin et al. 1994).

There are marked differences in the levels of plasma catecholamines observed in different species in response to different kinds of stress. The resting concentration of catecholamines in teleosts is generally between 1 and 5nM.L^{-1} (Randall and Perry 1992) which is similar to hagfish (Perry et al. 1993; Bernier et al. 1996). The maximum levels reported for hagfish are less than in teleosts and are at the low end of the range of concentrations of adrenergic agonists I have tested on veins. Perry and colleagues (Perry et al. 1993) reported the highest published concentrations of NA measured in the blood of *Myxine glutinosa* (during anoxia) of approximately 2x10^{-8}mol.L^{-1} or 20nM.L^{-1}. These workers observed higher levels of catecholamines (40nM.L^{-1}) in an *in situ* saline perfused heart preparation, following the application of high concentrations of potassium to the perfusate (60mmol.$L^{-1}K^{+}$). In Pacific hagfish *E. stoutii*, undergoing hypoxic treatment, Bernier and colleagues measured a maximal increase in plasma NA to $\sim$ 10nM.L-1 with

little change in AD levels.

It is possible that the physiological range for catecholamines *in vivo* in *E cirrhatus* extends further than reported for other hagfish species, as can be seen from the following chapter (Chapter 4). In this work I measured very high levels of circulating catecholamines during stress. In blood samples taken during heavy anaesthesia and surgery, plasma catecholamine concentration could exceed 1000nM.L^{-1}. It is unlikely that the "normal" physiological range for catecholamines in *E. cirrhatus in vivo* would extend to 1000nM.L^{-1}as surgery represents an extreme and un-physiological stress. However, the range may extend into hundreds of nanomoles as values in this range were measured during volume manipulation experiments in the following chapter. Significant venomotion of the veins occurred in this range in the myography experiments and this provides further support for possible *in vivo* modulation.

3.4.2 Adrenergic Receptors and Their Interactions

It is important to have an understanding of the actions of the catecholaminergic agents and their interactions with adrenoceptors.

The observation by Dale (1905) that the contractile effect of adrenaline was reversed by ergotoxin into a dilatation was a first step leading to the discovery of adrenoceptors. An explanation for this was not apparent until many years later when Ahlquist (1948) noted two patterns when certain adrenergic agonists caused pharmacological responses in a series of organs. As a result, Ahlquist introduced the concept of two distinct types of adrenergic receptors as defined by their responses to NA, AD and ISO. α-receptors were defined as those that responded in rank order of agonist potency as AD>NA>>ISO whilst β-receptors responded in order of ISO>AD>NA.

$\alpha-$adrenergic receptors are responsible for vasoconstriction whilst β receptors elicit vasodilatation. In this isolated *in vitro* preparation, the potentially confounding effects of most other systemic vasoactive substances are absent. Pressor and depressor responses seen in these isolated experiments are best explained by direct stimulation of α and β receptors present in the veins. However, even in this preparation it is possible that other vasoactive substances may be released provided there are stores of that substance present in the tissue and the appropriate secretory stimuli are applied (Busse and Fleming 2003).

Currently there are nine known adrenoceptor subtypes with two other possible candidates, although these may prove to be conformational states of existing subtypes (Guimaraes and Moura 2001).

Because vessels may contain both types of adrenoceptor, the net response to agonists that stimulate both types of receptor (e.g. the endogenous catecholamines) depends on the relative importance of each receptor population, as can be seen from the following example taken from Guimaraes and Moura (2001). In the dog saphenous vein, *in vitro* AD causes vasoconstriction, which is enhanced by β adrenoceptor blockade (Guimaraes 1975); in the rabbit facial vein, AD elicits vasodilatation, which is enhanced by α adrenoceptor

blockade (Pegram et al. 1976). Conversely, the contractile response of the saphenous vein to AD is converted to a dilatation when an α adrenoceptor antagonist is present (Guimaraes and Paiva 1981), and the relaxation to AD in the rabbit facial vein is changed to a vasoconstriction in the presence of a β adrenoceptor antagonist (Pegram et al. 1976). Thus in the dog saphenous vein the α adrenoceptor mediated influence dominates, whilst in the rabbit facial vein the dominating influence is exerted by the β adrenoceptor. These opposing effects exerted by differential receptor populations are the likely explanation for the highly varying responses of the veins to AD (see sections on individual vein types).

3.4.3 Vasoactivity of *E. cirrhatus* Veins to Adrenergic Stimulation

As previously mentioned, we chose to forgo testing complete groups of veins with the endogenous compounds in favour of the pure agonists, which gave clear, non ambiguous responses (see ISO and PHE myography). It was, however, important to establish whether the vessels were able to respond to the endogenous substances. Work on the vasoactivity of hagfish veins to adrenergic stimulation showed that all were capable of reacting vasoactively to both endogenous and exogenous catecholamines.

3.4.4 Anterior Cardinal Veins

The cardiovascular system of hagfishes has major differences from those of other vertebrates (see corrosion casts Chapter One). The two anterior cardinal veins (ACVs) are relatively large bore conduits, which drain blood from the anterior regions of the animal back to the portal (right ACV) and systemic hearts (left ACV). For consistency, we employed left ACVs in this study, which are of interest as they return blood to the systemic heart and are well positioned to influence this return through venomotion.

Veins were capable both of vasoconstriction and vasodilatation, though the response was modest in ACVs (<30mg tension change figure 3.1, 3.4 and 3.7), with a number of viable veins that failed to react at all. And so, although ACVs are capable of reacting, they do not appear be able to produce potent responses. It is not known how strong venomotion would have to be to mediate physiological effects *in vivo*, or even if modulation of venomotion in ACVs by catecholamines is important, however this work has shown venomotion of ACVs is possible, at least *in vitro*.

Limited conclusions may be drawn from these preliminary data, however, it appears as though these veins maybe more sensitive to AD than to NA as veins responded to lower, more physiological levels of AD. The high threshold (1000nM) for the constrictor response seen in the one ACV that did respond to NA, is far in excess of previously reported maximum circulating values in other hagfish species (Perry et al. 1993; Bernier et al. 1996).

The venoconstrictor response of Fish 1's ACV diminished at the highest concentrations of NA administered and had reduced to baseline levels at the highest dose.

Although it is possible desensitisation (tachyphylaxis) occurred at high NA concentrations, addition of the non selective β antagonist sotalol, showed that opposing vasodilatory β adrenergic stimulation was responsible for the decrease in α-adrenergic tone. The addition of sotalol returned the venoconstriction to almost peak levels.

Exposure of ACVs to AD produced contradictory responses, vasodilatation from Fish 1 and constriction in the vein from Fish 2.

The responses to the pure adrenergic agonists PHE (α) and ISO (β) were unambiguous (figure 3.4) compared to the responses to the endogenous catecholamines. Even though responses at all doses were not always significant in all veins examined, PHE exerted a pressor response and ISO a depressor response, as seen with the arteries of teleost species.

ACVs were particularly sensitive to both exogenous agonists (EC_{50} values were similar; figure 3.4b). β-adrenergic stimulation produced a significant decrease in tension, this proving to be the most potent action in terms of absolute change. The data further support the capacity of the vessels to react to adrenergic modulation, as demonstrated for the branchial vasculature of *Myxine glutinosa* (Axelsson et al. 1990).

Due to the lack of replicates it is difficult to say what the normal response of isolated ACVs is to endogenous catecholamines, but the responses indicate that NA might induce venoconstriction and AD vasodilatation at measured concentrations (see Chapter Four).

Though it has negligible affinity for β-adrenergic receptors, PHE is not as potent an α-agonist as either NA or ADR in teleost or elasmobranch fish (Holmgren and Nilsson 1974; Nilsson et al. 1975; Forster 1981) and thus it is not possible to draw conclusions about differential sensitivities of α- and β- adrenergic receptors based on the EC_{50}s for PHE and ISO.

It is not known what ability the ACVs, or any of the veins tested, have to respond *in vivo* but even a small change in vascular tone/compliance can affect stressed volume and therefore venous return (Olson 1998; Pang 2001). As ACVs (and the other veins tested) are large capacitance vessels they have the potential to affect stressed volume. However, peripheral vessels will also have a major effect on the stressed blood volume: mean circulatory filling pressure and venous return are mediated by the peripheral vasculature (Rothe 1986). Cardiac function will also influence central venous pressures, especially in animals that utilize *vis-a-fronte* filling.

Of all the vessels tested, ACVs were the least responsive in terms of magnitude of response (figure 3.7) with a proportion showing no response at all to adrenergic stimulation (it should be noted that these vessels were considered viable as they did respond to carbachol. However, their response to this agonist was depressed when compared to the viable vessels that did respond vasoactively to catecholamines). It cannot be determined from this work whether the depressed activity is due to a reduced receptor population, less amplification in the secondary signalling pathway, poor endowment of vascular smooth muscle or if this vessel just has a lower threshold for mechanical damage during vessel collection than the other veins investigated.

It is of course possible that the ACV is regulated by control systems other than humoral

catecholamines. However, although we found this vessel to be responsive to a range of other vasoactive agents, it was still the least responsive of the three veins to all substances tested.

3.4.5 Posterior Cardinal Veins

The two posterior cardinal veins (PCVs) of *E. cirrhatus* are homologous to the single vena cava in more advanced vertebrates. The paired veins collect venous blood from regions posterior to the systemic heart and blood is returned to the systemic heart via the sinus venosus (the collecting area and pacemaker of the hagfish heart) (Davie et al. 1987). The veins run in parallel in close proximity with a number of venous anastamoses, providing bypasses between the two vessels, connecting the two veins along their length (see corrosion casts Chapter One). The dorsal aorta is anchored between the two great veins with the venous anastamoses passing over the ventral surface of the artery. The left PCV has the larger calibre of the two veins and was used for this work.

The results of pharmacological studies on catecholamine effects in PCVs revealed (like ACVs) these veins also respond with active venomotion.

Again NA caused a potent venoconstriction, likely mediated via α adrenoceptors, with little evidence of β stimulation. This activity (figure 3.2a) became significant at a relatively high concentration (1000nM) which may be due to NA being stored in this vessel. This was also apparent as the EC_{50} was equivalent to 1370nM showing the PCV is not particularly sensitive to NA. The potency in the physiological range (i.e. less than $1x10^{-6}molL^{-1}$) was similarly low (<10mg increase in tension) irrespective of the stronger venoconstriction induced at higher concentrations.

It is known that in hagfish both the systemic and portal hearts and the PCV can release catecholamines (Bloom et al. 1961; von Euler and Fange 1961; Perry et al. 1993), thus it is more likely in this vessel than in the others we investigated, that local concentrations of endogenous catecholamines would be high enough to mediate a significant paracrine action of NA.

Whilst NA produced only constrictor responses in the PCVs, It was shown again that AD could induce both pressor and depressor activity. Taken together with the depressor response elicited by the pure β agonist ISO, it appears that AD was acting on β adrenoceptors present in the PCV to elicit vasodilatation .

This dilatation (figure 3.2b) occurred at low concentrations within the physiological range and only a very weak (< than 5mg) biphasic constrictor response was observed at the highest doses. One PCV responded to AD administration (Fish 2) with constriction only, however this response was weak and occurred at high, non-physiological concentrations. Although we had limited replicates for AD responses, the data support the theory that β mediated venodilatation may be physiologically more important in this vein.

The potent and significant effects of the pure agonists (PHE and ISO) on PCVs supported observations that endogenous catecholamines were most likely acting on α and β

adrenoceptors, confirming a reasonable population of both adrenoceptors in this vein.

3.4.6 Superior Intestinal Vein (SIV)

The SIV is a single large calibre vein which receives venous blood from the gut and splanchnic circulation and directs this flow to the portal heart.

As with the previous veins investigated, SIVs responded to catecholaminergic stimulation vasoactively and they exhibited the most potent action of all the vessels investigated. Potent venomotion in SIV's was concentration-dependent, attaining changes in baseline tension greater than 50mg for the endogenous agonists and greater than 100mg for exogenous compounds.

The responses in SIV were qualitatively similar to those observed in the other veins tested. ISO and PHE reactivity confirmed the capacity for pure α and β adrenoceptor mediated activity.

NA caused only vasoconstriction whilst AD elicited both pressor and depressor activity. The array of activity to AD shows a great variability in the capacity of these veins to react and could provide a finely tuned control *in vivo* dependent on the physiological needs of the animal. It is possible that receptor population (as in other veins) in this vessel may be labile accounting for the very different responses observed. This also supports the concept that in hagfish, as in other vertebrates NA has substantially greater α than β activity as only constrictor responses were observed. The endogenous catecholamine AD, elicited equipotent vasodilation and constrictor responses with no obvious bias.

SIVs were the only vessel in which we routinely observed spontaneous activity, which has been reported in elasmobranches and other vertebrates (Olson et al. 2000). Spontaneous activity occurred in these vessels in response to stretch or an increase in tension whether it be manual (i.e. setting the vessel to baseline tension between 300-400mg) or due to the action of a vasoconstrictor substance. It is possible endogenous stores of vasoactive agents are contained in this vessel and could be released by stretch. That SIVs exhibit the most potent but not the most sensitive response suggests possible greater secondary signalling amplification or smooth muscle mass.

3.4.7 General Comments:

As we have shown, the PCVs of *E cirrhatus* clearly have the ability to vasodilatate with sufficient β adrenergic stimulation (Figure 3.2b and 3.5a). One must conclude that NA has a greater capacity for α-adrenergic stimulation in these vessels as this substance only produced vasoconstriction.

The location of the veins we have investigated is interesting as they feed either the systemic heart or the portal heart. Changes in venous capacitance has the potential to regulate venous return to these hearts *in vivo* by altering preload (Olson1998).

The systemic heart receives inputs from the PCV, left ACV and the liver (Forster et al. 1991; Johnsson et al. 1996). Although it has been shown that hagfish do not require

a portal heart for survival (Davison 1995), return to the branchial heart from the liver is influenced by the portal heart and associated vasculature.

Johnsson and colleagues (1996) have shown in *E. cirrhatus*, that although portal heart rate was not altered by increased afterload, stroke volume, cardiac output and power output were reduced, with power output plateauing at an afterload of 0.22kPa (or 2.24cm water). *In situ*, total output from the portal heart fell at output pressures above ambient, but this might be a consequence of cutting connective tissue around the heart which otherwise allows *vis-a-fronte* filling. These workers also demonstrated that portal heart rate is tonically stimulated by catecholamines acting on β adrenoceptors. Infusion of the β blocker sotalol decreased portal heart rate whilst adrenaline administered as a bolus injection accelerated it. The portal heart contains substantial subendocardial stores of endogenous catecholamines, of which NA is the predominant type (von Euler and Fange 1961). These catecholamines not only have the potential to modulate the portal heart itself, but the downstream vasculature of the liver thus impacting venous return to the systemic heart. The portal heart itself is supplied by the SIV (and the right ACV, although we have not surveyed this vessel and know nothing about its activity). Preload has also been shown to be important in the modulation of the portal heart in *E. cirrhatus*. Increased input pressure (SIV pressure) led to an increase in cardiac output, stroke volume, power output and portal heart rate (Johnsson et al. 1996). Return to the portal heart has the potential to be controlled by the SIV which we have shown to be a particularly active vessel often displaying spontaneous activity in vitro (personal observation). Similarly the PCV may also offer a point of control as it has been shown to be reasonably responsive to catecholamines, and is the major conduit for the flux of blood back from the periphery.

3.4.8 Conclusion

Surveys of blood vessels from a limited number of "phyletically ancient" chordates indicate that the vertebrates are remarkably conservative in the expression of receptors for agents involved in cardiovascular control (Evans, 2001; Evans and Harrie, 2001; Simpson et al., 2000; Sundin et al., 1994). This suggests these mechanisms arose early in chordate evolution, at least 500 million years ago, when the hagfishes diverged from the chordate stock (Bardack, 1998; Shu et al., 1999).

Research has shown that catecholaminergic regulation as a method of physiological control has been conserved and components of this system are present in the phyletically ancient hagfish (Reite 1969; Axelsson et al. 1990; Forster et al. 1991; Forster et al. 1992; Perry et al. 1993; Sundin et al. 1994). Much of this work has focused on the modulatory effects of catecholamines in controlling the branchial arterial vasculature, during hypoxia or anoxia.

Until now, there has been no specific investigation into the pharmacology of catecholamines on the venous vasculature of hagfish, most likely due to the traditional view that active venomotion in fish was an unlikely phenomenon. This work shows unequiv-

ocally, that the veins of *Eptatretus cirrhatus* have the capacity to react to endogenous and exogenous catecholamines. Adrenergic modulation of the venous vasculature could be important physiologically as it has the potential to alter venous return via alterations in systemic capacitance.

Chapter 4

In Vivo Catecholamines in *Eptatretus cirrhatus*

4.1 Introduction

The catecholamines noradrenaline (NA) and adrenaline (AD) are released in fish in response to a variety of internal and environmental stressors (Nilsson 1983; Nilsson 1984; Schreck 1990). Studies have also shown that catecholamines are important in the normal cardiovascular function of fish (Wood and Shelton 1980; Nilsson 1994; Zhang et al. 1998a). The control of catecholamine storage and release in the chromaffin cells of teleosts is a complex process involving regulation by a variety of neurotransmitters, hormones and second messenger systems (Reid et al. 1998). The adrenergic responses of teleosts are the most well investigated and understood with few studies that have investigated the *in vivo* adrenergic responses of hagfishes (Perry et al. 1993; Bernier et al. 1996).

In view of the lack of *in vivo* studies on the adrenergic responses in hagfishes and the vasomotion of *E cirrhatus* veins to adrenergic stimulation (Chapter Three), experiments were performed to measure plasma catecholamine concentrations during various manipulations.

Anaesthesia and surgery can have marked effects on the cardiovascular system of fish, they are often associated with hypoxia and acid-base disturbance and increases in catecholamines may occur as a result (Gingerich and Drottar 1989; Iwama and Mcgeer 1989; Hill and Forster 2004). Because animals underwent surgery and anaesthesia to implant cannulae for blood sampling and blood pressure measurement it was important to characterise associated adrenergic responses.

Volume manipulation studies in fish provide excellent models to investigate acute and tonic vascular control systems (Duff and Olson 1989; Zhang et al. 1998b; Minerick et al. 2003; Olson et al. 2003). Studies on the effects of volume manipulation on cardiovascular parameters were carried out in previous work (Chapter Two). Similar experiments were performed to assess the adrenergic responses of *E. cirrhatus* to these manipulations and to see if adrenergic responses correlated with changes in cardiovascular parameters observed in the earlier work .

Surgery and anaesthesia, and volume manipulation experiments all elicited significant changes in basal plasma catecholamine concentrations. Surgery and anaesthesia and volume loading experiments evoked similar responses to those observed during hypoxia in other species of hagfish where increases in plasma NA predominate (Perry et al. 1993; Bernier et al. 1996). Volume loading experiments caused decreases in basal NA and concomitant increases in AD that appear to be adaptive physiological responses in view of the opposing adrenergic responses observed during volume depletion.

4.2 Methods

4.2.1 Experimental animals

The New Zealand hagfish, *Eptatretus cirrhatus* Forster, was used in all experiments. Hagfishes were collected off Motunau beach or Akaroa harbour, New Zealand and were transferred to the University of Canterbury in Christchurch where, they were held in aquaria containing circulating sea water. A total of 33 animals were used for this work with a mean weight of 1108±52g (S.E.M.). Animals were kept at 12-14 $^{\circ}$C under a 12 hour light: dark cycle. They were held at least one week prior to experimentation, and were not fed during this period.

4.2.2 *In Vivo* Catecholamines during Anaesthesia and Surgery

Hagfish were blood sampled during surgery, recovery and at rest to ascertain what effect these treatments had on plasma catecholamine concentrations (NA and AD).

This protocol was carried out in fish undergoing cannulation surgery for preliminary work for this thesis. The surgical method is the same as described in the methods section for Chapter Two.

Once animals had reached the required depth of anesthesia, at approximately 60min, they were transferred to an operating table and surgery was commenced. Prior to instrumentation with cannulae, a 0.4ml blood sample was collected directly from the posterior cardinal vein. After surgery animals were placed in individual holding tanks and a steady flow of aerated seawater was passed across the gills by means of a tube inserted into the nasal opening until the animals regained consciousness.

Once animals had regained consciousness (between three and five hours after initial anesthesia) a second 0.4ml blood sample was collected from the venous cannula (PCV). To prevent contamination, samples were retrieved from cannulae using a two syringe method. 1ml of blood was withdrawn from the cannula into a first syringe, and then the 0.4ml sample was drawn into a second, new syringe. The initial 1ml of blood was re-injected and the cannula was flushed through with 0.4ml of heparinised hagfish saline.

Sampling was repeated the following morning whilst the animals were at rest. Samples were treated, stored and assayed by HPLC for catecholamines, in the same manner as the serial sampling experiments described later in this methods section.

Haematocrits were also calculated for some samples. Blood was drawn into capillary tubes and spun in an Eppendorf centrifuge at 3000rpm for 5 minutes to separate plasma and red cells. The total length of the sample in the capillary tube and the length occupied by red cells was measured. The haematocrit was calculated as the percentage of red cells present in the total sample.

4.2.3 *In Vivo* Resting Concentrations of Catecholamines

To define more comprehensive resting concentrations of NA and AD in *E. cirrhatus*, data were compiled from 16 animals. This included some resting data from both of the following sections; *in vivo* catecholamines during anaesthesia and surgery and *in vivo* serial blood sampling during volume manipulation. The compiled data also included data not featured elsewhere in this thesis.

Blood samples were carefully collected from resting undisturbed animals, one day after cannulation surgery. Samples were rejected for analysis if animals became disturbed during collection. Blood was collected from either the PCV or the DA via indwelling cannulae using the two syringe method. Samples were treated, stored and assayed by HPLC for catecholamines in the same manner as in the serial sampling during volume manipulation experiments described below. Haematocrits were also measured in some of these samples.

4.2.4 *In Vivo* Serial Blood Sampling During Volume Manipulation

Hagfish were blood sampled during volume loading and depletion to ascertain what effect these treatments had on plasma catecholamine concentrations (NA and AD). These experiments replicate volume manipulation experiments presented in Chapter Two, with the difference that blood samples were collected for catecholamine analysis and blood pressures were not recorded during this time.

Surgical Method

Hagfish were anaesthetized in seawater containing MS222 (ethyl-*p*-aminobenzoate and 3-aminobenzoic acid ethyl ester metanesulfonate 0.4g L^{-1}) and benzocaine (0.4g L^{-1}). After approximately 60min, the fish were transferred to an operating table where a small mid ventral incision was made to expose and allow cannulation of the dorsal aorta.

Dorsal aortae were cannulated non-occlusively with Portex polythene tubing (0.86mm ID, 1.27mm OD) which was, in turn, connected to a length of larger diameter polythene tubing (1.0mm ID, 2.0 OD). The open ends of cannulae were advanced towards the systemic heart. Cannulae were threaded through a small cuff of latex rubber, which was secured to surrounding tissue with superglue to hold cannulae in place. Cannulae contained heparinised, hagfish HEPES buffered saline (HHBS) of the same composition used previously (see Methods section Chapter Two).

Volume Manipulation

After 24 hours of recovery post surgery, animals were either volume loaded or depleted. Volume manipulation was achieved by turning off the recirculating seawater supply to the tank, followed by the addition of a measured quantity of either distilled water or dissolved sea salt to change the salinity of the tank water from 100%, to approximately 90% or

110% seawater. The tank water was continuously aerated with an air stone, once the recirculating seawater supply had been switched off.

The tank water was slowly changed to the experimental medium (∼110% or ∼90% seawater) 1-2 minutes before time 0 to allow a gradual introduction of the new medium over 2 minutes for all volume manipulation experiments.

Once the first volume manipulation and serial sampling experiment had been completed, animals were given 24 hours to acclimate to the changed experimental medium (either ∼110% or ∼90% seawater). Following the 24hr acclimation period the experimental medium was switched back to 100% seawater, thus representing a volume manipulation opposite to that performed 24 hours previously (i.e. if animals were switched back to 100% from a 90% acclimation medium, then they experienced volume depletion). Serial sampling experiments were also performed during the "switching back" phase and these data were pooled with data collected for the same type of manipulation during the initial volume change to reduce the number of animals required to generate data sets.

Serial Sampling

Blood samples to test for catecholamine levels and osmolarity were collected at 20 minute intervals, while tank water samples for osmolarity were collected at the beginning and end of each volume manipulation experiment. Blood sampling commenced 20 minutes prior to volume manipulation (control sample) and continued at 20 minute intervals until 100 minutes had elapsed post volume manipulation. Blood sampling was performed using the two syringe method however, as blood samples were collected every 20 minutes, cannulae were not flushed with 0.4ml of heparinised saline after every sample. Each preparation acted as its own control for investigating the differences in catecholamine levels after volume manipulation via osmotic stress.

Blood samples were spun down using an Eppendorf centrifuge at 3000rpm for 2 minutes to separate plasma. 5μl of 2M glutathione and EDTA were added to 100μl of plasma and samples were then stored at -80 $^{\circ}$C until they were assayed for catecholamine concentration by HPLC (within 1 month of collection). The remaining plasma was retained for measurement of osmolarity.

NA and AD concentrations were determined in alumina extracted plasma samples using high performance liquid chromatography and electrochemical detection (Woodward 1982). Known quantities of NA and AD were used as standards at the beginning and end of each set of samples assayed by HPLC (Forster et al. 1998).

Plasma and seawater osmolarities were measured using a Wescor vapour pressure osmometer, calibrated against standards.

Results from this work showing changes in blood osmolarity and confirming that fish were exposed to an approximate 10% change in salinity to produce volume manipulation, are presented in Chapter Two (figure 2.1).

4.2.5 Effects of Exogenous NA on *In vivo* Pressures

Some preliminary work was carried out in which exogenous NA was injected into the PCV of *E.cirrhatus* and venous and aortic pressures were monitored.

1ml of $1x10^{-4}$M NA was administered via the PCV cannula and was flushed through with 0.5ml of heparinised hagfish saline. Prior experiments had shown negligible pressurizing effects occur as a result of the injection of 1.5ml of heparinised hagfish saline into the PCV.

Pressures in the PCV and DA were recorded via indwelling cannulae in the same manner as described in the experiments presented in Chapter Two. Pressures were averaged for 5 minutes preceding NA administration and for 10 minutes following this treatment.

4.2.6 Statistical Analyses

Statistical analyses used are stated in figure legends.

Due to unequal variance between data sets, non parametric tests were used where appropriate. T tests or their non parametric equivalents were used in favor of ANOVA for analysis of serial sampling during volume manipulation experiments because a comparison between the pre-change control value and individual, post volume manipulation time points was required.

All statistical tests were two tailed unless otherwise stated and all means are presented ±S.E.M. The limit of significance was $P<0.05$.

4.3 Results

4.3.1 *In Vivo* Catecholamine Concentrations During Surgery and Anaesthesia in *E. cirrhatus*

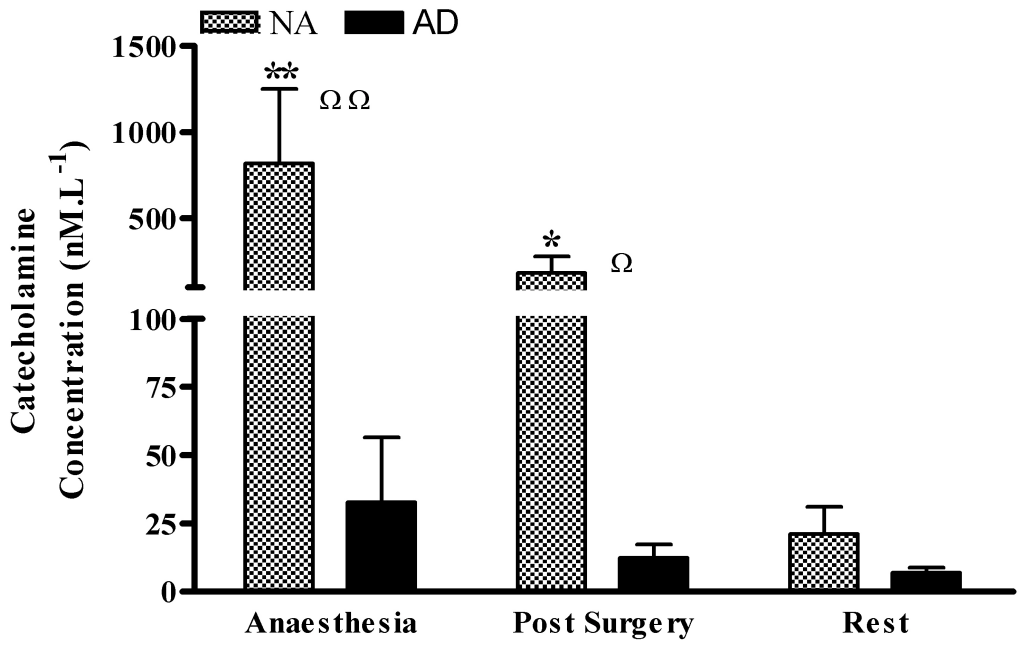


Figure 4.1: *In vivo* catecholamine concentrations whilst anaesthetised during surgery (n=9), after surgery when animals had regained consciousness (n=6) and at rest on the day following surgery (8). All data are presented as the mean ±S.E.M. * over bars indicates catecholamine levels that are significantly different to the corresponding resting value for that catecholamine, taken at rest 1 day after surgery. $^{\Omega}$ between bars indicates that the concentrations of the two catecholamines measured, are significantly different for that treatment (Mann-Whitney U test, *or $^{\Omega}$ $P<0.05$, ** or $^{\Omega\Omega}P<0.001$)

Both NA and AD concentrations were measured in the same samples collected during the treatments shown in figure 4.1.

Anaesthesia and surgery evoked a large and significant release of NA compared to resting levels of NA, and levels of AD evoked by the same treatment. NA was clearly the predominant catecholamine released with an average ratio of approximately 25:1nM.L^{-1} NA to AD (note split scale on the Y axis). The mean concentration of NA during anaesthesia and surgery for 8 fish was 818 nM.L^{-1}. Owing to the large variation in the responses of individual animals, the standard error was high at 432nM.L^{-1}. The range of the response was also large with the lowest plasma NA concentration measuring 42nM.L^{-1} and the highest 3587nM.L-1. Of the 9 fish tested, 6 released noradrenaline in excess of 200nM.L^{-1}. Concentrations of plasma AD also appeared elevated in some fish during surgery, with a mean concentration of 33 ± 24nM.L^{-1}, however this was not a significant

difference when compared to resting values. The range of AD concentrations in venous blood during anaesthesia and surgery was between 0 and 199nM.L^{-1}.

NA levels were still significantly elevated and significantly different to AD after surgery once these animals had regained consciousness. Although the mean NA concentration post surgery was lower, it was not significantly different to NA levels measured during surgery. The mean NA concentration for 6 fish was 185 ±94 nM.L^{-1}. Post surgery AD concentrations were not significantly different to resting concentrations, with a mean value of 12 ±5 nM.L^{-1}. The ratio of NA to AD was lower than during surgery, but still in favor of NA with ∼ 15nM.L^{-1} NA to 1nM.L^{-1} AD.

Levels of NA had decreased significantly by the following day to a mean resting concentration of 21 ±10nM.L^{-1}. The mean concentration of AD for these same samples was 7 ±2nM.L^{-1} and there was no significant difference in the concentration of the two catecholamines at this time.

4.3.2 *In Vivo* Resting Concentrations of Catecholamines and Haematocrit

Data from sixteen animals were compiled to obtain resting concentrations of NA and AD in *E. cirrhatus*. The mean NA concentration was 6.85 ±2.07nM.L^{-1} and the mean for AD was 23.85 ±12.79nM.L^{-1} (±S.E.M.). Although the AD value is nominally higher, the variance is also higher and the results of a paired T test showed no significant difference between the resting concentrations of the two catecholamines ($P<0.05$).

A resting haematocrit was calculated for nine of these animals and the mean red cell haematocrit was 11.38 ±0.94% (±S.E.M.). Haematocrits were also measured in six of the nine fish that underwent surgery and anaesthesia. The mean hematocrit of venous blood samples collected in this manner, was 12.49 ±0.77% (±S.E.M.). There was no significant difference between the samples collected at rest and during surgery (Unpaired T test, $P<0.05$).

4.3.3 Serial Blood Sampling During Volume Manipulation

NA Volume Depletion

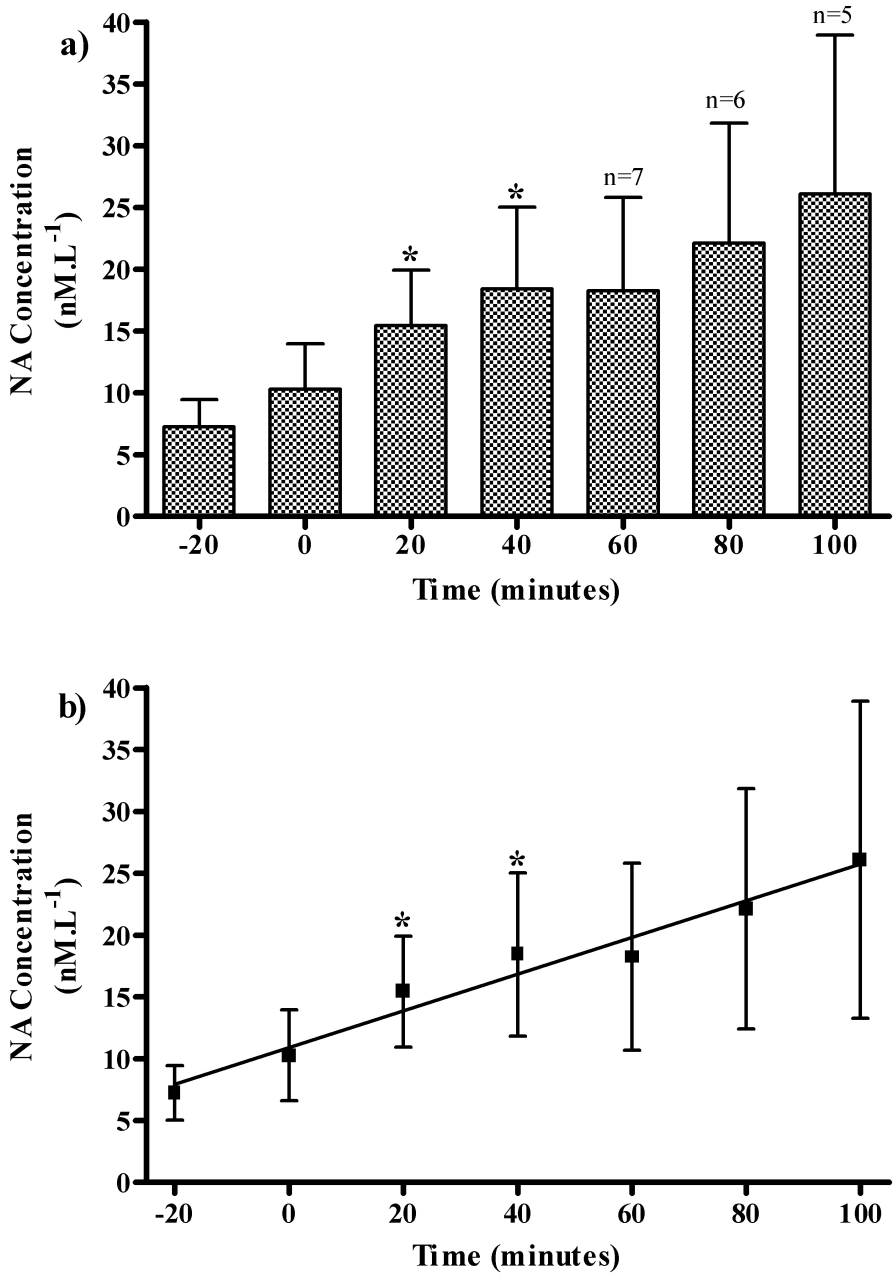


Figure 4.2: Plasma NA levels during serial blood sampling volume depletion experiments in *E. cirrhatus*. The number of replicates varied between 5 and 8 as the full complement of samples was not always able to be collected at each time point. All data are presented as the mean ±S.E.M. a) n values different to n=8 are noted above the corresponding bars and * indicates time points where concentrations of NA were significantly different to baseline concentrations at -20 minutes (Wilcoxon matched pairs test, $P<0.05$). b) A highly significant linear regression could be fitted to the data presented in 2a), $P<0.0001$ with a positive slope value of 0.15 ±0.01.

Non parametric analysis was used as there was considerable variability in the data, as can be seen in figure 4.2. The Wilcoxon matched pairs test detected significant increases (compared to baseline concentrations at -20 minutes) at 20 (P=0.0078) and 40 minutes (P=0.0156). At -20 minutes or baseline, the mean concentration of NA was 7±2nM.L^{-1}. By 20 minutes this had increased approximately 2 fold to an average of 15 ±4nM.L^{-1} and by 100 minutes this had increased further to 26nM.L^{-1} although the S.E.M. was large at 13nM.L^{-1}.

With linear regression analysis, the slope of the line was significantly different to zero and indicated a rise in NA concentrations over time.

Paired analyses could not be applied to the data after the 40 minute time point due to missing values.

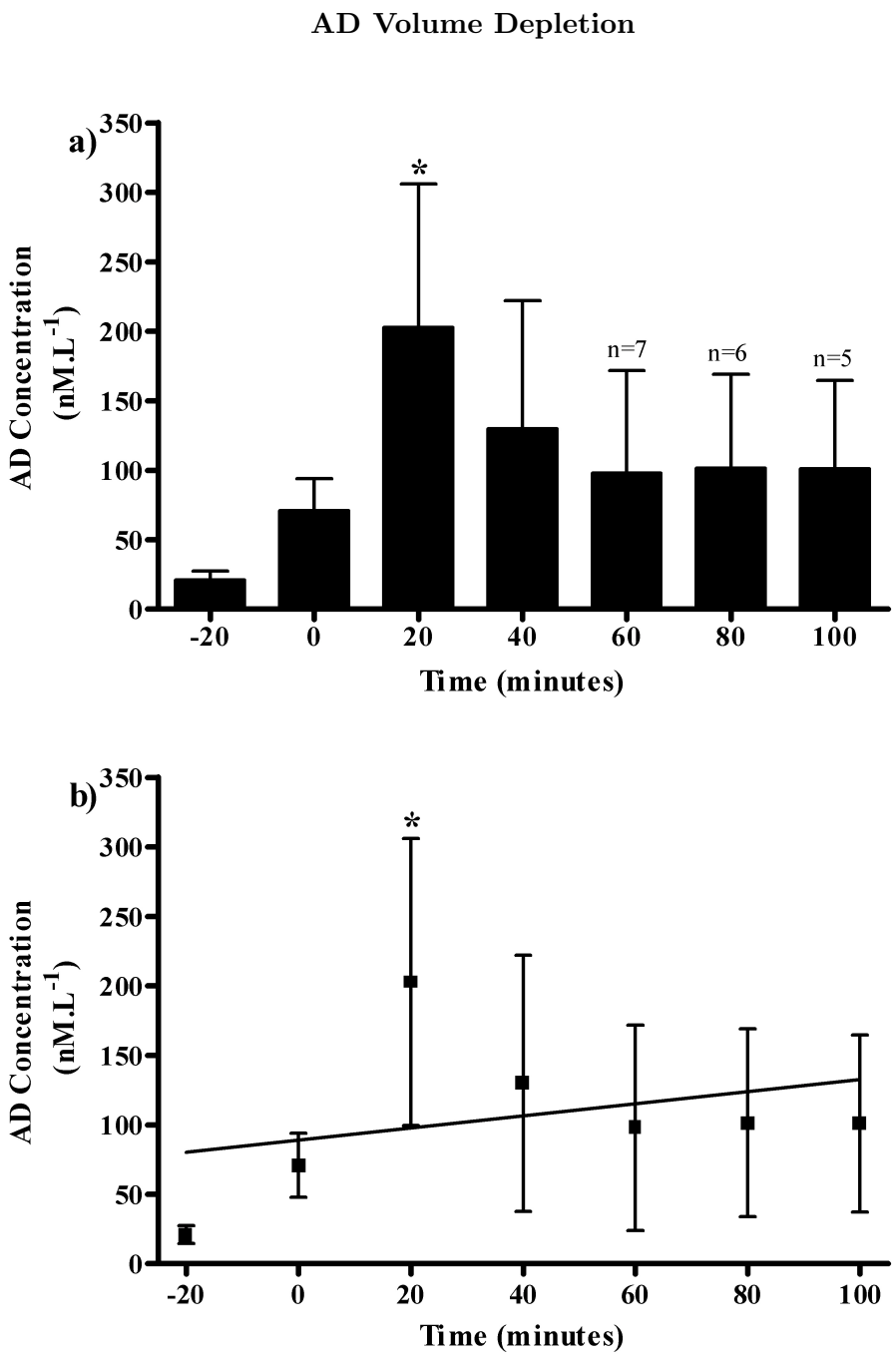


Figure 4.3: Plasma AD levels during serial blood sampling volume depletion experiments in *E. cirrhatus*. n varied between 5 and 8 as the full complement of samples was not always able to be collected at each time point. All data are presented as the mean ±S.E.M. a) n values different to n=8 are noted above the corresponding bars and * indicates the time point where concentration of AD was significantly different to baseline concentration at -20 minutes (Wilcoxon matched pairs test, P=0.0313). b) With linear regression analysis (2a) there was no significant deviation from 0 ($P<0.05$).

A surge in AD release occurred at 20 minutes and was significantly different from baseline concentrations, however this response declined and the concentration was not significantly elevated above pre-change values by 40 minutes (P=0.074).

The mean baseline concentration of AD was 21 ±6nM.L^{-1}, this increased quickly to 71 ±23nM.L^{-1} at 0 minutes and then to 202 ±103nM.L^{-1} by 20 minutes.

The mean concentration decreased to 130 ±92nM.L^{-1} at 40 minutes and then stabilised to approximately 100nM.L^{-1} for the last three time points (98 ±74nM.L^{-1} at 60minutes, 101 ±68nM.L^{-1} at 80 minutes and 101 ±64nM.L^{-1} at 100 minutes).

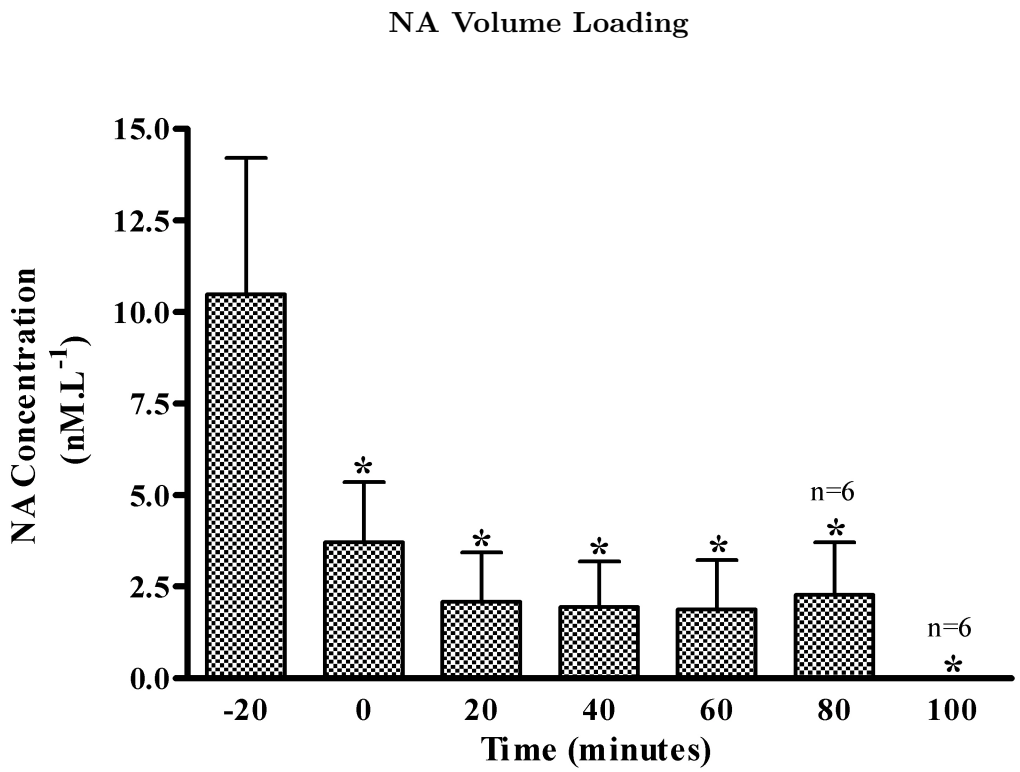


Figure 4.4: Plasma NA levels during serial blood sampling volume loading experiments in *E. cirrhatus*. The number of replicates varied between 6 and 7 as the full complement of samples were not always able to be collected at each time point. n values different to n=7 are noted above the corresponding time points. All data are presented as the mean ±S.E.M. * indicates time points where concentrations of NA were significantly different to baseline concentrations at -20 minutes (0 to 60 minutes Wilcoxon matched pairs test, 80 minutes one tailed Mann-Whitney U test, 100 minutes One sample T test *P<0.05).

Statistical testing revealed a significant effect of volume depletion on NA levels at all time points. NA concentrations were seen to decrease immediately on induction and remain decreased for the full 100 minutes of the experiment. This was particularly notable at 100 minutes when no NA could be detected in any of the samples collected from six fish.

This is supported by the application of linear regression to the data, which gives a negative trend line significantly different to zero (P=0.0016, slope=-0.06 ±0.02, graph

not shown). The mean pre-change concentration was 10 ±4nM.L^{-1} this decreased to 4 ±2nM.L^{-1} at time 0 and then to 2 ±1nM.L^{-1} at 20 minutes, after this point values stayed approximately the same with the same error until 100 minutes when no NA was detected.

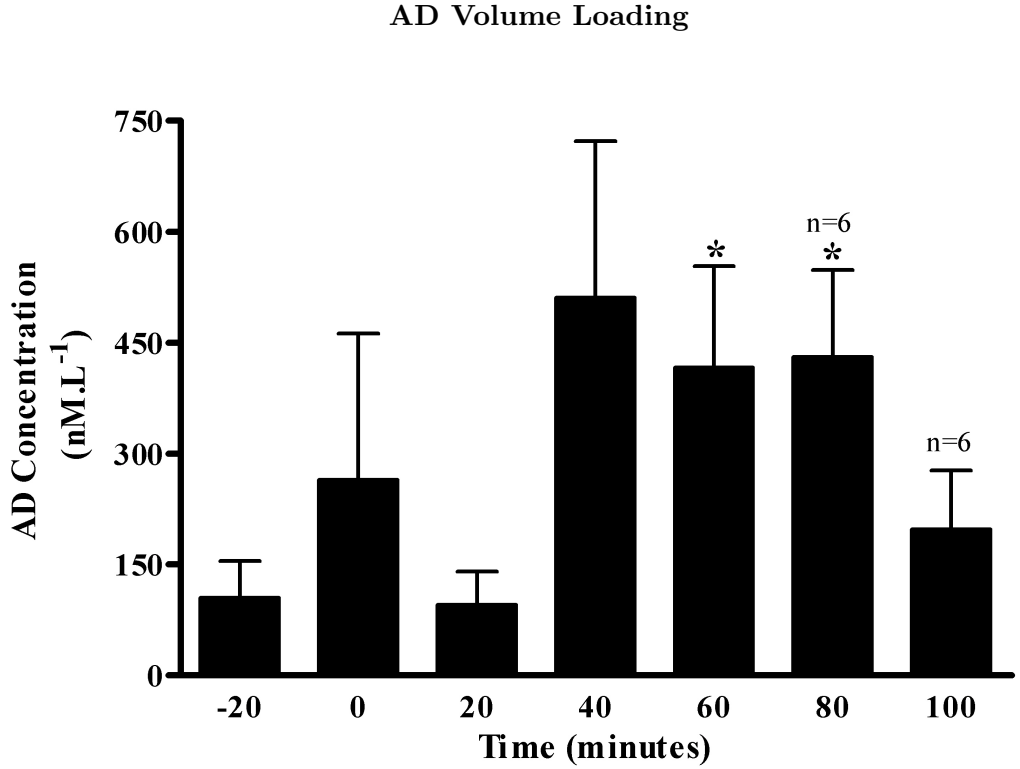


Figure 4.5: Plasma AD levels during serial blood sampling volume loading experiments in *E. cirrhatus*. The number of replicates varied between 6 and 7 as the full complement of samples were not always able to be collected at each time point. n values different to n=7 are noted above the corresponding time point. All data are presented as the mean ±S.E.M. * indicates time points where concentrations of AD were significantly higher than baseline concentrations (60 minutes= one tailed Wilcoxon matched pairs test, P=0.0391 and at 80 minutes= one tailed Mann Whitney U test, P=0.0367).

The variance of the AD response to volume loading was different between time groups and so non parametric analysis was applied. The results of non parametric ANOVA (Kruskal-Wallis test) although not significant, approached significance with a P value of 0.073 and the results of the appropriate non parametric tests detected significantly higher AD concentrations at 60 and 80 minutes than compared to pre-change values. Regression analysis of the data showed no trend line significantly different to zero.

Baseline, pre-volume loaded AD concentration was higher than in the volume depletion experiment, with a mean of 105 ±50nM.L^{-1}. Mean increases in AD peaked at 40min with a concentration of 511 ±212nM.L^{-1} and this approached significance with a P value of 0.0781 (one tailed, Wilcoxon matched pairs test). At 60 minutes the mean AD concentration was 416 ±137nM.L^{-1} and at 80 minutes it was 430 ±118nM.L^{-1}, this decreased at 100 minutes to 197 ±80nM.L^{-1}.

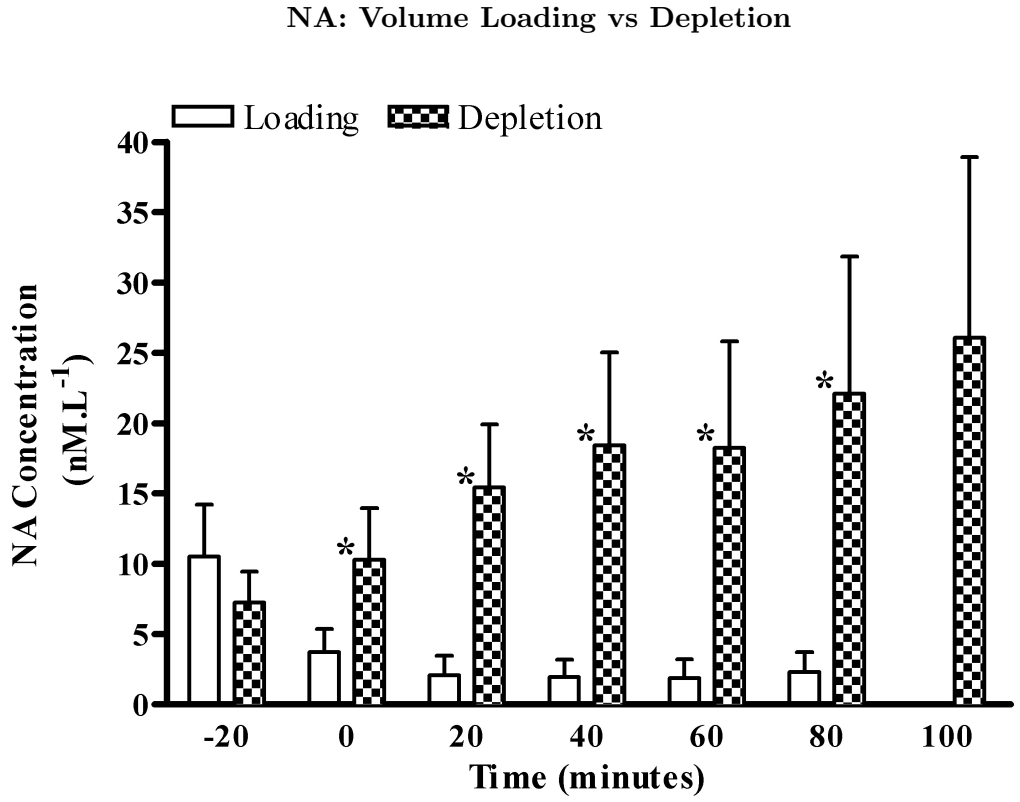


Figure 4.6: Plasma NA concentrations during serial blood sampling experiments compared at each time point for differences in NA concentrations between volume loading (figure 4.4) and depletion (figure 4.2). All data are presented as the mean ±S.E.M. * Between bars indicates time points where levels of NA were significantly different between volume loading and depletion (Mann-Whitney U test, $P<0.05$, note the test at 0 minutes is one tailed).

Volume manipulation caused significant differences in the concentration of NA. Between the time points of 0 to 80 minutes, levels of NA were significantly higher in the volume depleted animals. The 100 minute time point could not be tested for significance as the mean for volume depletion was 0 ±0nM.L^{-1}.

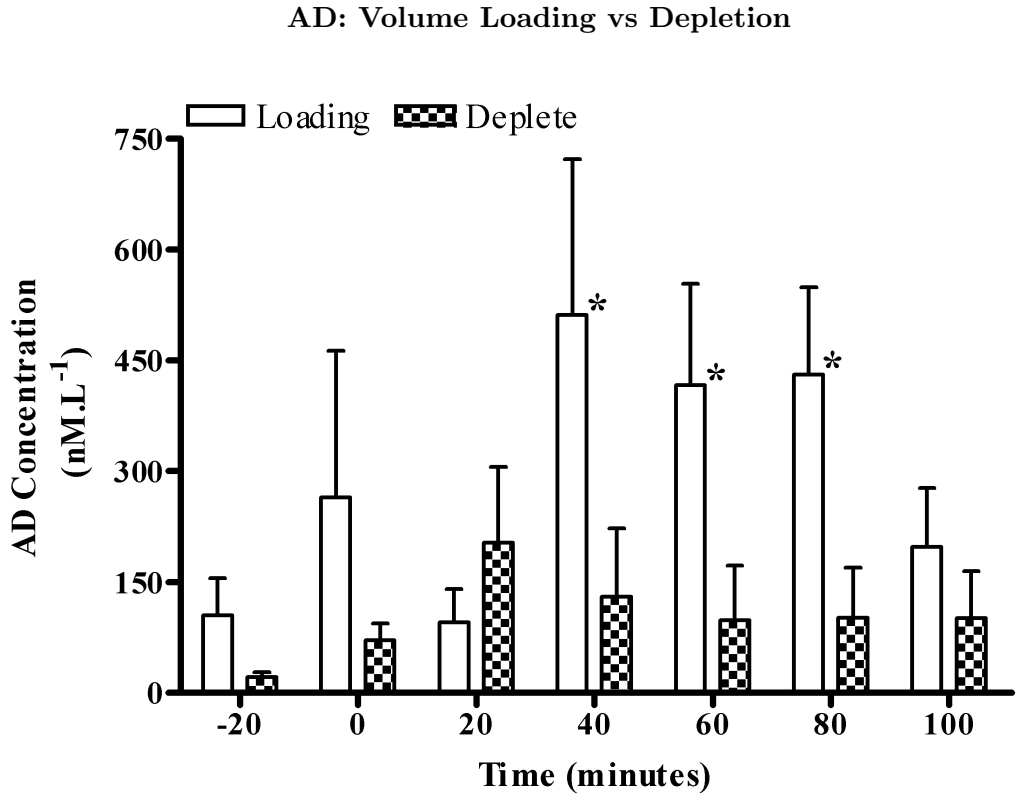


Figure 4.7: Plasma AD concentrations during serial blood sampling experiments compared at each time point for differences in AD concentrations between volume loading (figure 4.5) and depletion (figure 4.3). All data are presented as the mean ±S.E.M. * Between bars indicates time points where levels of AD were significantly different between volume loading and depletion (Mann-Whitney U test, $P<0.05$, note the test at 80 minutes is one tailed)

Volume manipulation caused significant differences in the concentration of AD. Between the time points of 40 to 80 minutes, levels of AD were significantly higher in the volume loaded animals.

4.3.4 Effects of Exogenous NA on *In Vivo* Pressures

Some preliminary work was carried out to determine if application of exogenous NA directly into the PCV would affect venous and aortic pressures. The mean PCV pressure for five fish in the 5 minutes preceding this treatment was 0.56 ±0.65 cmH_2O and the mean PCV pressure for the 10 minutes following the bolus of NA was 0.92 ±0.54cmH_2O. Although there was considerable variation in the data, pairing was significantly effective in controlling for this and the results of a paired T test revealed a significant difference between pre and post NA administration PCV pressures (one tailed, $P=0.0391$). There was an average increase in PCV pressures of 0.36cmH_2O after NA administration.

Only two of these fish were monitored for DA pressures, however they both displayed increases in pressure in response to exogenous NA administration. In the 10 minutes

following treatment, the mean DA pressure increased by 3 cmH_2O in one fish and by 1.80 cmH_2O in the other.

Heart rate did not seem to be affected appreciably by this treatment. One fish had a resting heart rate of 18.21BPM which increased by 1 beat per minute to19.21BPM, the other animal had a resting rate of 18.51BPM which changed very little to 18.38BPM.

4.4 Discussion

4.4.1 *In Vivo* Catecholamine Concentrations During Surgery and Anaesthesia

Surgery and Anaesthesia

For experimental research it is common practice to surgically implant cannulae to provide an easily accessible port to the circulation of an animal. This kind of access is typically used when repeated blood sampling is required and may also be used for the measurement of intravascular pressures, as has been done in experiments on the blood pressure responses of *E. cirrhatus* (volume manipulation experiments Chapter Two).

This method of blood sampling minimises animal handling during sampling and reduces the associated stress, however it does require animals to undergo anaesthesia and surgical manipulation which, in itself can pose a severe stress to animals (Perry and Bernier 1999). Cannulation was used extensively here, and given the focus of this work on catecholamines and their effects in *E. cirrhatus*, it was important to evaluate the effects of surgery and anaesthesia on *in vivo* catecholamines.

Anaesthesia and surgery evoked a significant release of catecholamines in the hagfish, a response which has also been documented in other fish species receiving similar treatment (Le Bras 1982; Gingerich and Drottar 1989; Iwama and Mcgeer 1989).

Because catecholamine release is a common response to stress in fish, it is difficult to attribute a specific causative stimulus for this response (Reid et al. 1998). It is likely that the additive effects of pre surgery handling, prolonged anaesthesia with associated hypoxaemia and acid-base disturbance and the trauma of surgery all contributed to the huge adrenergic outflow measured in *E. cirrhatus* during surgery and during recovery in this study.

Combinations of these stressors may have additive effects, Hill et al (2004) found a prolonged recovery time in Chinook salmon that underwent surgery and anaesthesia in comparison to fish that underwent anaesthesia alone. They also found marked effects of pre-surgery handling that masked anaesthetic dependent effects on the cardiovascular system of these fish. Netting prior to anaesthesia caused marked increases in heart rate (HR), cardiac output (CO) and stroke volume (SV), all responses which may have been mediated by release of catecholamines.

Having said this, there is substantial evidence that one of the most important triggers for catecholamine release in teleosts and cyclostomes is hypoxaemia with other factors such as acid base status modulating the extent of the response, (Boutilier et al. 1986; Tang and Boutilier 1988; Perry et al. 1993; Perry and Reid 1994; Bernier et al. 1996; Julio et al. 1998; Reid et al. 1998; Reid and Perry 2003; Perry et al. 2004). It is likely hypoxaemia played an important role in mediating the adrenergic response to surgery and anaesthesia in *E.cirrhatus*.

Resistance to Anaesthesia

Local anaesthetics used in mammals such as MS222 and benzocaine are the most commonly used anaesthetics for fish. These agents enter the circulatory system of fishes via the gills and are generally rapidly taken up and distributed when dissolved in the environmental medium (Meinertz et al. 1991).

In comparison to other fish species, hagfish were particularly resistant to anaesthesia and required extended exposure (∼60 minutes) in comparatively strong anaesthetic, to achieve a depth suitable for surgery. When placed in anaesthetising media (seawater containing dissolved MS222 and benzocaine) hagfish would often respond with a short burst of vigorous swimming followed by the rapid cessation of ventilation and swimming. Although animals stopped ventilating early in anaesthesia (<10 minutes) they were still responsive to touch until an extended period of exposure had elapsed.

It is possible that this "breath holding" behaviour is a nociceptor mediated response to the anaesthetic, that may limit the uptake of anaesthetic into the circulation. Nociceptors, are receptors located on fish gills that respond to noxious stimuli, when stimulated they elicit bradycardia, hypotension, shallow respiration and a brief cessation of swimming (Satchell 1991; Sundin and Nilsson 2002). The physiological responses evoked by nociceptors are thought to reduce uptake across the gills and slow transport from the gills to the tissues (Satchell 1991). It is also possible that the early cessation of ventilation may be due to a direct effect of the anaesthetic on the velum, the structure responsible for the ventilatory current.

Although *E. cirrhatus* may not be exposed to hypoxic/anoxic conditions in its natural environment to the same degree as its burrowing Atlantic cousin *M. glutinosa*, they are still likely to experience periods of low environmental oxygen availability similar to the period of ventilatory arrest that they experienced during anaesthesia. Animals are potentially exposed to hypoxia when feeding as hagfish may burrow into their prey and when defending themselves as they secrete copious amounts of slime which often surround the animal compromising ventilation (Martini 1998). *E. cirrhatus* may not withstand hypoxia as well as *M. glutinosa* (Forster et al. 1992), however, they still possess various physiological features which adapt them to this situation better than the majority of teleosts (Davie et al. 1987; Forster et al. 1989; Davison et al. 1990; Forster 1990; Forster et al. 1991; Forster 1998).

This episodic exposure to low environmental oxygen availability coupled with adaptive physiological features such as their low metabolic rate, high anaerobic capacity of the heart and large blood volume make "breath holding" a viable strategy which minimises anaesthesia uptake once the animals become aware they are unable to escape the noxious stimulus in their environment. How effective this breath holding behaviour is at minimising internal concentrations of anaesthesia might be questioned in view of the high permeability of hagfish (Rudy and Wagner 1970). Indeed, rapid changes in plasma osmolarity were measured during the volume manipulation experiments supporting high permeability in

this species. During volume manipulation there was no observable cessation of ventilation. This may have facilitated a greater permeability to the external environment than may occur when ventilation has ceased and there is likely to be a greater flux across the gills. During anaesthesia transport via the gills would be decreased if hagfish exhibit a nociceptor mediated response similar to that of teleosts which could depress circulation reducing the uptake and transport of anaesthetic molecules in the blood. How this would occur in an animal with an apparent aneural heart (Fange et al. 1963*b*) is unclear as an increase in vagal tone may elicit bradycardia in teleosts during nociceptor mediated responses (Satchell 1991). To elicit such a response hagfish would have to reduce their CO and although HRs are slow typically 20-30 beats per minute, CO is comparable to other inactive lower vertebrate species (Forster et al. 1991). Having said this, volume manipulation work has shown that hagfish have the ability to reduce central venous pressure (Chapter Two) which may be influenced by circulating catecholamines. A reduction in central venous pressure would almost certainly reduce CO, however as blood pressures were not measured during early anaesthesia it is unknown if this occurs.

Anaesthetic molecules crossing the epithelium of the animal would first encounter the subcutaneous sinus of the animal, a capacious blood sinus which separates the skin from the body wall and extends over most of the body. Forster et al (1989) demonstrated that a period of at least 8 hours was necessary to get thorough mixing of central and sinus blood in *E. cirrhatus.* Another feature of hagfish physiology that may contribute the observed resistance to anaesthesia is the comparatively large blood volumes of these animals (McCarthy and Conte 1966; Forster et al. 1989). Hagfish have the largest blood volumes reported among the vertebrates. With an apparent 30% of the blood in the subcutaneous sinus it is possible that this compartment could act as a sink for anaesthetic molecules so as to extend the time to reach the critical internal threshold for induction of anaesthesia. In view of the previous comments regarding slow turn over between the compartments, this is unlikely to provide a sink for the central circulation. And so with apnoea, slow turn over of sinus blood and possible cardiovascular regulation the central circulation of the hagfish may be protected to some degree from the entry of anaesthetic.

Cardiovascular function during Anaesthesia

Anaesthetics such as MS 222 and benzocaine do not affect cardiovascular parameters when used locally, however, local anaesthetics depress cardiovascular function markedly if they enter the blood stream in sufficient quantity (Stoelting and Miller 1989). Common cardiovascular effects of anaesthesia can include; bradycardia and arrhythmias, decreases in blood pressure (BP), and reductions in cardiac output (CO) and fishes must compensate for these effects if they are to maintain adequate cardiovascular function (Randall 1962; Peirce and Peirce 1967; Houston 1971; Lochowitz et al. 1974; Fredricks et al. 1993).

Previous studies suggest that a fall in dorsal aortic pressure (DAP) is a general response to MS 222 anaesthesia in other fish taxa (Randall et al. 1965; Peirce and Peirce 1967;

Fredricks et al. 1993; Hill and Forster 2004). Hill and Forster (2004) found that salmon treated with MS 222 exhibited a continuous decline in DAP but showed little change in HR, CO or stroke volume (SV) suggesting a direct vasodilatory effect of this agent. Using *in vitro* myography Hill et al (Hill et al. 2004) found MS 222 acted as a potent vasodilator of the branchial arteries of salmon and reduced contractility of paced strips of ventricular myocardium by 75%.

Direct effects of anaesthesia have not been studied in hagfish although it is likely that effects on the cardiovascular system are similar to those in teleosts, and that the increase in plasma catecholamines may counteract some of these deleterious effects.

An acute adrenergic response under these circumstances may help to maintain cardiovascular homeostasis as catecholamines can stimulate increases in CO via compensatory changes in HR and stroke volume (SV) (Axelsson et al. 1990). Catecholamines have been shown to influence a plethora of cardiovascular parameters in fish such as HR, cardiac contractility, vascular resistance, functional blood volume, haematocrit and oxygen carrying capacity of the blood (Boutilier et al. 1986; Axelsson et al. 1990; Forster et al. 1992; Johnsson and Axelsson 1996; Johnsson et al. 1996; Reid et al. 1998; Crocker et al. 2000; Agnisola et al. 2003) Catecholamines may exert their influence directly on the vasculature to minimize cardiovascular perturbations caused by anaesthesia. As an effector of the sympathetic nervous system (responsible for mediating adrenergic responses in higher vertebrates) NA is well known to have a role in regulating peripheral vascular resistance through alterations in venomotor tone (Pang 2001). Additionally in an *in vivo* study on trout, (Zhang et al. 1998) has shown that catecholamines have potent effects on veins. The application of AD was shown to mobilise blood from the unstressed into the stressed compartment and augment central venous pressure by reducing venous compliance. Alteration of the venous compartment may be particularly important in the face of such challenges as the greater proportion of blood volume resides here and alterations in venous tone and compliance provide a rapidly acting mechanism for the compensatory redistribution of blood volume (Rothe 1986)

Cardiovascular function during Hypoxaemia

Hypoxaemia is an indirect effect of anaesthesia seen in all animals when anaesthesia compromises ventilatory function. Studies on fish show a low Pao_2 associated with anaesthesia followed by a quick recovery once ventilation is resumed (Soivio et al. 1977; Iwama and Mcgeer 1989; Hill and Forster 2004).

In this study PO_2 was not measured in blood samples but it is a fair assumption the animals were hypoxaemic for the following reasons; 1) the anaesthetising medium was not oxygenated, so passive diffusion across the epithelium would not contribute to oxygenation of the blood, 2) the burst of activity upon induction of anaesthesia would likely deplete oxygenation of the blood and could create an O_2 debt, 3) there had been no ventilation of the gills for approximately 60 minutes prior to sample collection and 4) arterial blood

appeared deoxygenated during surgery, as it was dark purple in colour which was in strong contrast to the bright red samples obtained from DA cannulae when animals were at rest.

Hypoxemia provides a cardiovascular challenge and physiological function may be altered to improve the efficiency of gas exchange, transport of oxygen in the blood and delivery to the tissues so that cellular respiration is not compromised (Boutilier et al. 1988; Fritsche and Perry 1989; Perry and Thomas 1991). The physiological actions of an acute humoral adrenergic stress response support cardiovascular function and facilitate appropriate cardiovascular responses to such challenges as hypoxaemia.

Maintenance of homeostasis in hagfish during cardiovascular disturbances brought about by the direct effects of anaesthesia and the indirect effects of hypoxaemia and attendant acid-base disturbance would require the activation of a counteracting system such as the sympathetically mediated adrenergic response often seen in teleosts. It is likely the huge surge in plasma NA measured during surgery and anaesthesia in *E. cirrhatus* had a functional homeostatic role.

That catecholamines have the capacity to influence cardiovascular function in hagfish has been shown by a number of studies. In an *in situ* preparation of the perfused portal heart of *E. cirrhatus*, the application of sotalol (a β-adrenergic blocking agent) caused a significant decrease in HR, whilst bolus injections of AD produced transient increases in HR, indicating the presence of an endogenous β-adrenergic tonus on the portal heart (Johnsson et al. 1996). In similar work on the systemic heart of *M. glutinosa*, sotalol produced a comparable decrease in HR and power output was reduced by 40% (Johnsson and Axelsson 1996). In *M. glutinosa* injection of AD into the caudal vein significantly increased CO, SV, HR, and blood pressure in the DA and ventral aorta (VA) (Axelsson et al. 1990). In *E. cirrhatus* injection of AD elicited similar responses with increases in HR, SV and arterial blood pressures and decreases in systemic and branchial resistance (Forster et al. 1992).

Evidence that catecholamines are likely to have had an adaptive role in the cardiovascular challenges that *E. cirrhatus* was exposed to during in this study, are provided by studies of the cardiovascular responses of hagfish exposed to hypoxia.

Forster et al (1992) measured a 40% increase in the CO of *E. cirrhatus* with a severe decrease in the partial pressure of oxygen in the environmental medium (Pwo_2= 5.3kPa). Significant increases in the plasma NA of the reputedly more hypoxia tolerant species *M. glutinosa* and *E. stouti* occurred at lower environmental oxygen partial pressures(Pwo2= 1.4kPa and Pwo2= 1.33kPa respectively)(Perry et al. 1993; Bernier et al. 1996) .

The increase in CO was attributed to increased SV as there was little change in HR. As the animals were more active during hypoxic exposure, the increase in CO may have been partially due to physical activity boosting venous return and increasing SV, however CO continued to increase to 160% of control values during recovery when animals were inactive suggesting some mechanism, other than skeletal muscle contractions was responsible for the continued response.

Since teleost fish hearts have an end systolic volume close to zero, the end diastolic

volume is the major determinant of SV (Farrell 1991; Forster and Farrell 1994). Because of this, major increases in SV cannot be obtained by increasing cardiac contractility despite the fact that catecholamines have been shown to mediate positive inotropic effects (Axelsson et al. 1990). With inotropic effects unlikely to evoke substantial increases in SV, and with no change in HR in the Forster et al (1992) study, the only explanation for the observed increase in CO is an increase in cardiac filling and therefore end diastolic volume. *In vitro* the hagfish heart responds to increased filling pressure by increasing SV in accordance with the Frank-Starling mechanism (Chapman et al. 1963; Forster et al. 1991). Previous work in this thesis (Chapter Two) does not support the existence of *vis-a-fronte* filling of the systemic heart of *E. cirrhatus* and so it is most probable that increases in cardiac filling occur because of augmented venous return.

As with *E. cirrhatus* undergoing surgery and anaesthesia, the animals in the Forster et al (1992) study were exposed to a severe hypoxic stress and it is probable that this caused a release of catecholamines from the animals' endogenous stores (chromaffin tissue). This adrenergic response could mediate an increase in venous return by acting on the vasculature to rapidly mobilise blood into the stressed compartment. Sandblom and Axelsson (2005) measured increases in central venous pressure during hypoxia in Rainbow Trout and hypothesise that an active increase in venous tone serving to mobilize blood into the central venous compartment is an important cardiovascular trait associated with hypoxia.

As systemic resistance did not change in the Forster et al (1992) study, venous return in hypoxic *E. cirrhatus* cannot have been boosted by blood mobilised from the periphery. This is not surprising as the adaptive response to lowered oxygen saturation of the blood is vasodilation at the tissues, to maximize perfusion and ensure efficient gas exchange. Infact, it has been suggested that a decrease in peripheral resistance (a common response to hypoxia in fish) may increase cardiac preload by reducing the pressure gradient between the arterial and venous circulations with a resultant increase in central venous pressure and a decrease in DA pressure (Sandblom and Axelsson 2005).

In view of previous findings that NA mediates significant increases in tension of central veins via α adrenoceptor stimulation (catecholamine myography Chapter Three), that the central venous vasculature is a significant storage site of NA in hagfish (Perry et al. 1993) and that *E. cirrhatus* has been shown to release significant amounts of NA during hypoxic stress, I would argue that an α-adrenoceptor mediated increase in central venous tone, is a likely mechanism to increase cardiac preload during hypoxia and anaesthesia in these animals.

Although the traditional view was that active control of venous tone in fish was unlikely (Satchell 1991), there is growing evidence that this is not the case and that it may be an important cardiovascular control mechanism *in vivo* in fish (Conklin et al. 1997; Olson et al. 1997; Zhang et al. 1998; Hoagland et al. 2000; Sandblom and Axelsson 2005).

There is good evidence that branchial vascular resistance in hagfishes is under tonic adrenergic control (Axelsson et al. 1990; Forster et al. 1992; Sundin et al. 1994). That branchial vascular resistance dramatically increased in *E. cirrhatus* during hypoxia

(Forster et al. 1992), also suggests an increase in α-adrenergic stimulation as the gills are situated immediately downstream from the catecholamine stores in the cardinal veins and hearts of hagfish.

In a similar study on *M. glutinosa* Axelsson et. al. (1990) found CO was maintained and that there were significant elevations in DA and ventral aortic (VA) pressure during and after hypoxic exposure of 1.5 to 2.2 kPa. Perry (1993) has shown significant release of NA at similar levels of hypoxia in this species and it is likely that this contributed to the maintenance of CO during hypoxia in *M. glutinosa.*

Potency of Response

The average absolute concentration of plasma NA in *E. cirrhatus* undergoing surgery and anaesthesia was far in excess of that measured previously in other hagfish species during stress. Although there was considerable variation in the levels released by individuals, even the smallest response (to 42nM.L^{-1}) was approximately double that of the highest reported value measured in any other hagfish species for NA (Perry et al. 1993).

Circulating levels of NA could exceed 1000 nM.L^{-1} in hypoxic *E. cirrhatus*, however they only reached an average of 20 nM.L^{-1} in anoxic *M. glutinosa* (Perry *et al.*, 1993). *M. glutinosa* burrows into soft sediments, where as *Eptatretus* species live on rocky substrates in crevices rather than burrows (Strahan 1963; Forster 1990). *Myxine* survives in anoxic conditions, but *E. cirrhatus* struggled violently in attempts to escape a closed respirometry box once the PO_2 had fallen below 5 kPa (Forster 1990). The greater response in *E. cirrhatus*, as measured by catecholamine release, might therefore reflect a greater sensitivity to hypoxia in this species when compared to *M. glutinosa.* Axelsson et al (1990) found that a reduction in PO_2 of the environmental media of *M. glutinosa* to 4kPa had no effect on cardiovascular variables and a further reduction to less than 2.2kPa was required to elicit cardiovascular responses similar to those seen in *E. cirrhatus* at an environmental PO_2 threshold of 5kPa (Forster et al. 1992).

Variations in the individual responses of *E. cirrhatus* to anesthesia and surgery could be attributed to a number of factors such as condition of the animals prior to surgery, preconditioning due to prior stressful events, duration of anaesthesia etc. There is likely to be a relationship between severity of the treatment and the potency of the response. Regardless of the variation, this work (combined with the adrenergic responses to volume manipulation) has shown these animals are capable of substantial catecholamine release, greater than previously recorded for hagfish.

Dichotomy of Catecholamine Response

NA was the most abundant catecholamine secreted with no significant change in plasma AD concentration. The predominance of plasma NA over AD seen during surgery and anaesthesia in *E.cirrhatus* has also been reported for other hagfish species undergoing similar physiological challenges. Perry et al (1993) found that hypoxaemia in *M. glutinosa*

was associated with a significant increase in circulating NA with no significant change to plasma AD and Bernier et al (1996) found a similar effect in the Pacific hagfish *E. stouti*. This, combined with the findings presented here, suggests that NA release may be a common response to hypoxia in hagfish.

As can be seen from the volume manipulation experiments, *E.cirrhatus* is capable of significant AD release. That this does not occur during hypoxia and anesthesia and surgery suggests that of the two catecholamines, NA is likely to be better suited to eliciting appropriate adaptive responses to hypoxia such as α-adrenoceptor mediated increases in central venous tone. AD may not be as effective in eliciting the appropriate responses to these challenges as it appears to have greater β stimulatory ability than NA (in the central veins of *E. cirrhatus* at least). AD could conceivably have deleterious effects on venous return via β-adrenoceptor mediated dilatation of the central venous vasculature. However, with myography Forster(1998) showed that AD and NA were equipotent in stimulating vasoconstriction of afferent and efferent branchial arteries from *E. cirrhatus*, and so caution is required when extrapolating *in vivo* effects from *in vitro* work.

It has been suggested that an abundance of circulating NA maybe a primitive trait, as it has been reported only in lower vertebrates such as elasmobranchs, cyclostomes and also in invertebrates. In teleosts and higher vertebrates AD is generally the most abundant catecholamine released into the blood (Randall and Perry 1992; Perry et al. 1993; Ottaviani and Franceschi 1996; Wendelaar Bonga 1997; Reid et al. 1998; Lacoste et al. 2001). This difference may reflect the greater evolution of the sympathetic nervous system in higher vertebrates, where NA is utilised primarily as a neurotransmitter and AD is released in to the circulation to mediate the acute humoral response to stress (Reid et al. 1998). This is supported by findings of Zhang (1998), where infusion of exogenous AD but not NA into trout increased venous tone and decreased venous compliance and inhibition of endogenous sympathetic nervous system activity had the same effect on tone and compliance in this teleost.

Predominance of circulating AD in higher vertebrates may also reflect an evolutionary up regulation in the enzymatic processes required for AD biosynthesis (Reid et al. 1998). Despite this hagfish are still capable of significant adrenergic responses utilizing AD as shown by the volume manipulation experiments and so ability to produce AD is not a limiting factor in their response to hypoxia.

Given the potency of catecholamines and the multiplicity of their targets, control over the magnitude and quality of the response to different kinds of stressors is crucial to the effectiveness of the response. The primary mechanism leading to the release of catecholamines from the chromaffin tissue in elasmobranchs and higher vertebrates is the stimulation of the chromaffin cells by preganglionic sympathetic fibers (Reid et al. 1998).

Although carbachol (an acetylcholine analogue) elicits catecholamine secretion in an *in situ* perfused heart preparation of *M. glutinosa* (Perry et al. 1993), this is not thought to be an important mechanism *in vivo* given the reputed absence of innervation of the chromaffin tissue in hagfish (Nilsson and Holmgren 1993).

The current evidence points to hormonal and or paracrine involvement of non-cholinergic pituitary factors in the control of catecholamine release in these primitive fish (Perry et al. 1993; Bernier and Perry 1996). Adrenocorticotropic hormone (ACTH) has been shown to be a potent secretagogue for catecholamines in hagfish (Perry et al. 1993; Bernier and Perry 1996). ACTH evoked significant increases in the secretion rate of both NA and AD in *M. glutinosa*, with an initial bias toward NA secretion (Bernier and Perry 1996).

Another secretagogue, serotonin has been shown to preferentially elicit NA secretion in *M. glutinosa* to an even greater extent than ACTH (Bernier and Perry 1996). There is immunohistochemical evidence for the presence of serotonin containing cells in the systemic and portal hearts of *M. glutinosa* (Reid et al. 1995). It has been suggested that these cells may be homologous to the neuroepithelial cells (NECs) ubiquitous in vertebrates (Bernier and Perry 1998). NECs are found in respiratory epithelia and are characterized by their ability to synthesise and store serotonin in chromaffin granules, and by their sensitivity to oxygen (Bailly et al. 1992). The possible chemoreceptor role of NECs in vertebrates may be shared by the serotonin containing cells of hagfishes. Exposure to hypoxia in fishes and mammals has been shown to decrease the number of NECs and increase exocytosis of their indolamine storing vesicles (Dunel-Erb et al. 1982; Lauweryns and Van Lommel 1982). Bernier and Perry (1998) suggest that in *M. glutinosa,* during severe hypoxia, serotonin may be released from stores in the systemic heart to mediate catecholamine secretion.

Bernier et al (1996) found that adenosine also appears to have a role in modulating the adrenergic response to hypoxia in hagfish. Plasma AD levels did not increase in Pacific hagfish exposed to hypoxia, however, significant increases were recorded in hypoxic *E. stouti* treated with an adenosine receptor antagonist (theophylline). Adenosine has been shown to have anti-adrenergic actions in various tissues and in mammals it modulates secretion of catecholamines from the adrenal medulla (Chern et al. 1987; Dobson et al. 1987; Mullane and Williams 1990; Chern et al. 1992; Tseng et al. 1994).

It is possible that these secretory and modulatory mechanisms may be involved in the dichotomous adrenergic response to hypoxia observed in *E. cirrhatus.*

Dichotomy in Storage of Catecholamines

Although catecholamine storage sites have not been investigated in *Eptatretus* species, work on myxinoids has shown the ratio of NA to AD varies considerably among different storage sites (Bernier and Perry 1998). The most abundant store is found in the systemic heart, followed by the portal heart and then the PCV (Ostlund 1954; Augustinsson et al. 1956; Ostlund et al. 1960; von Euler and Fange 1961; Perry et al. 1993). In the systemic heart the NA to AD storage ratio is close to 1, with the atrium containing mainly NA and the ventricle mainly AD (Ostlund et al. 1960; von Euler and Fange 1961; Perry et al. 1993). The portal heart and the PCV contain mainly NA (Ostlund et al. 1960; von Euler and Fange 1961; Perry et al. 1993).

Given their potential for activating either vasoconstriction or vasodilation it is inter-

esting that the distribution of AD and NA varies in its storage sites in *M. glutinosa*. The tissues holding venous return from the posterior end of the animal are in a position to respond to hypoxia with NA release, this local release could conceivably exert a paracrine effect on the central venous vasculature, which has been shown to vasoconstrict to physiological concentrations of NA (Chapter Three). Downstream effects of NA release from the portal heart have the potential to mobilize blood from the vast vascular bed of the liver, located between the portal heart and systemic hearts (Fange et al. 1963a). Venous outflow from the liver passes into the sinus venosus and is likely to have a marked effect on the total venous return.

The systemic hearts of hagfish receive no extrinsic innervation, thus the control of HR and SV depends on mechanisms other than the autonomic nervous system (Fange et al. 1963*b*). *In situ* perfused heart preparations in *M. glutinosa* show very little response to the application of exogenous NA and AD, however depletion of endogenous catecholamine stores in the systemic heart elicited marked bradycardia which was reversed with application of NA and AD (Fange and Ostlund 1954; Bloom et al. 1961). The existence of an adrenergic cardiac tonus can be further demonstrated by the severe bradycardia elicited by β adrenoceptor antagonists, such as sotalol (Axelsson et al. 1990; Johnsson and Axelsson 1996). It is therefore not surprising that the ventricle contains significant subendocardial stores of AD in view of its likely role in the adrenergic tonus on the heart.

What is interesting is that subendocardial stores of AD are not only in a position to mediate a paracrine adrenergic tonus on the heart but to exert their influence on the structures downstream from the heart. That the CO of the hagfish heart is particularly sensitive to afterload (Forster 1989; Axelsson et al. 1990; Johnsson and Axelsson 1996), and that the ventricle operates against the afterload of the branchial and systemic resistances points to a possible cardioprotective role of AD at high concentrations via β-adrenoceptor mediated modulation of downstream structures, though it vasoconstricted the major afferent and efferent branchial arteries at the lowest concentrations (Forster, 1998). This is supported by *in vivo* studies in hagfish where AD injection caused increases in CO that also increased DA and VA pressure but decreased systemic and in one study branchial resistance (Axelsson et al. 1990; Forster et al. 1992).

Although the specific storage sites of the two catecholamines have not been ascertained in *E. cirrhatus*, the dichotomous adrenergic response to surgery and anaesthesia and to volume manipulation supports the existence of separate populations of chromaffin cells containing the two catecholamines.

Post Surgery Recovery

E. cirrhatus were as slow to recovering from anaesthesia and surgery as they were at succumbing to anaesthetic induction. Some animals took up to five hours to regain consciousness. Such prolonged anaesthesia would present a severe homeostatic challenge that would require more than an acute adrenergic response to address the associated physio-

logical perturbations.

In light of the previous discussions it is not surprising that plasma NA levels were still significantly elevated during recovery as it is unlikely that cardiovascular homeostasis would be achieved as soon as animals regained consciousness. Studies of cardiovascular responses of hagfish to hypoxia show that cardiovascular changes persist into recovery and may even extend beyond those observed during the hypoxic exposure (Axelsson et al. 1990; Forster et al. 1992).

Regulation of HR and SV in hagfish are sensitive to venous filling pressure (Jensen 1961, Axelsson et al. 1990, Johnsson and Axelsson 1996, Johnsson et al. 1996). Venous volume loading by injecting of saline into healthy *M. glutinosa* produced increases in both SV and HR, as it did in apparently dying hagfish with abnormally low SV and pooling of blood in the subcutaneous sinus (Axelsson et al. 1990). Axelsson et al (1990) suggest that increases in venous filling pressure may be important after prolonged periods of anoxia and metabolic depression in the reactivation of cardiac activity, especially in view of the lack of cardiac innervation in hagfishes. These authors noted that vigorous sinusoidal tail movements (under neural control) that occur after anoxia/hypoxia may serve to activate the pressure sensitive pacemaker via increases in venous return.

Elevated NA levels observed in *E cirrhatus* recovering from anaesthesia and surgery are likely to augment venous return and may therefore influence a pressure sensitive pacemaker mechanism. However whether this mechanism exists in *E. cirrhatus* is uncertain, as an *in situ* perfused heart study found no change in heart rate with variation in preload (Forster 1989). As the preparation did not include the sinus venosus which is the pacemaker region *in vivo* (Davie et al. 1987), it may not have been able to respond to changes in preload.

4.4.2 *In Vivo* Resting Concentrations of Catecholamines and Haematocrit

The resting concentrations of catecholamines compiled from sixteen undisturbed *E. cirrhatus* were slightly higher than normal plasma concentrations reported for other species of hagfish. Bernier et al (1996) reported control values of 1.19nM.L^{-1} AD and 3.53nM.L^{-1} NA in the Pacific hagfish *E. stouti* and Perry et al (Perry et al. 1993) measured concentrations of 0.36nM.L-$_1$ AD and 1.8nM.L^{-1} NA in M. glutinosa. As the samples were taken in undisturbed animals it may be that basal levels are marginally higher in this species, although the large S.E.M. of the AD concentration suggests there may have been some unforeseen factor affecting AD levels in some fish.

It is possible that true resting values may be even lower than those measured in our samples, as they were collected one day post surgery in chronically cannulated fish. However as previously stated, collection of blood samples via cannula minimizes stress associated with sampling as it reduces the handling required for collection.

Additionally, studies on teleosts have shown plasma catecholamines to have reduced significantly by 24 hours after surgery and anaesthesia and suggest this recovery period

is adequate to allow estimation of resting plasma levels (Le Bras 1982; Gingerich and Drottar 1989). Minimisation of the recovery period in my experiments was a necessity as it allowed experimentation to commence as soon as possible after surgery. This was important due to the difficulty of keeping patent cannulae in these animals.

There were no significant differences between the basal concentration of the two catecholamines, which contrasts with the very different concentration of the two catecholamines during hypoxia, anesthesia and surgery and volume manipulation, and argues for a basal rate of release.

The resting haematocrit of 11.38% measured in this study is similar to 13.5% reported by Forster et. al. (1989) for the same species, and is comparable to haematocrits reported for other hagfish species. Bernier et al (1996) measured a resting haematocrit of 14.7% for *E. stouti* with Forster et al (2001) reporting a similar value of 14% in the same species. Perry et al(1993) measured a haematocrit of 11.2% in *M. glutinosa* but Toop and Evans (1993) measured a higher haematocrit for this species of 28%.

Teleosts may respond to hypoxia with an increase in haematocrit brought about by an adrenergically induced release of red blood cells from the spleen (Holmgren and Nilsson 1975; Yamamoto et al. 1985; Perry and Kinkead 1989). That haematocrit did not change in the samples collected during surgery is in agreement with reports of other workers who found hypoxia does affect haematocrit in hagfishes (Bernier et al. 1996). This correlates with the reported absence of a spleen in hagfish (Satchell 1991) and suggests they have no other comparable mechanism to increase circulating red cells in response to acute hypoxic challenge.

4.4.3 Serial Blood Sampling During Volume Manipulation

Serial blood sampling to measure plasma catecholamines during volume manipulation in *E. cirrhatus* indicated that catecholamines were significantly and differentially affected by volume loading and depletion.

Plasma Catecholamine Concentrations During Volume Depletion

The levels of plasma catecholamines showed significant changes during volume depletion experiments. While NA levels became significantly increased by 20 minutes with a continuing increasing trend, AD levels surged at 20 minutes but showed no continued trend.

The rapid increase in NA is predictable if this hormone is indeed released in response to a need to maximize cardiovascular function as seems to be the case during hypoxia. Although depletion is a different cardiovascular challenge than hypoxia, some similar alterations in physiological function are likely to be required to maintain homeostasis. Rather than affecting blood oxygen levels as in hypoxia, especially as this method of volume manipulation has been shown to concentrate the blood and increase haematocrit (Toop and Evans 1993), depletion threatens adequate perfusion of the tissues as stressed blood volume and therefore blood pressure is reduced. If NA affects the central venous vasculature

in vivo as it did in *in vitro* myography, then the α-adrenoceptor mediated pressor effects on the vasculature would be an adaptive response that could support CO by increasing venous return as may occur during hypoxia in the same species.

Bernier et al (1999) have shown that catecholamines are recruited in fish during hypotension. Intravenous papavarine injections (a smooth muscle relaxant) in the American eel and the rainbow trout induced marked decreases in aortic pressure. As a result there were significant increases in plasma catecholamines in both species.

The general lack of response of AD to depletion also seems similar to that observed in animals that were exposed to hypoxia during anaesthesia and surgery, as concentrations could not be distinguished from pre-change values except for an initial increase at 20 minutes. This initial surge had a mean concentration of 202nM.L-1 and is likely to have exerted a physiological effect at that time point, of which the significance is unclear.

Injection of exogenous AD *in vivo* in hagfishes can mediate increases in HR, SV and blood pressure (Axelsson et al. 1990; Forster et al. 1992). Given these effects it might be expected that this hormone may remain increased beyond the initial surge upon volume depletion. That AD levels were not significantly increased at later time points indicates sustained increases of this hormone may not be advantageous during volume depletion.

Although bolus injections of AD may influence HR, SV, and CO, how effective AD might be *in vivo* during volume depletion may be questioned due to: 1) the location of AD stores in the ventricle of the systemic heart downstream from the venous vasculature, 2) the greater β activity of AD than NA on central venous vasculature and 3) that changes in HR do not seem to be the major mechanism for influencing CO in hagfish (Forster et al. 1991). As a general rule, greater changes in CO are achieved via changes in SV rather than in HR, and SV is more dependent on end diastolic volume (which is determined primarily by cardiac filling) in teleost fish because end systolic volume is close to zero (Farrell 1991; Forster and Farrell 1994). This is supported by in vivo studies in hagfish that show changes in HR of no more than 25-40% were achieved as a result of hypoxia, exercise or agonist drug injections (Forster et al. 1988; Axelsson et al. 1990)

Even if increases in systemic heart rate could help maintain CO during volume depletion, it is questionable if increases in circulating AD could mediate such an effect. In contrast to the *in vivo* studies, *in vitro* studies on the isolated perfused heart of hagfish have shown little effect of adrenergic agonists on HR (Fange and Ostlund 1954; Ostlund 1954; Johnsson and Axelsson 1996). As isolated perfused hagfish hearts only become sensitive to adrenergic stimulation when their endogenous stores are depleted, it seems that endogenous catecholamine stores saturate cardiac β-adrenoceptors so that no greater adrenergic stimulation is possible (Bloom et al. 1961; Johnsson and Axelsson 1996).

It seems likley that increases in HR elicited by AD in the *in vivo* studies (Axelsson et al. 1990; Forster et al. 1992) may have been mediated by a pressure sensitive pacemaker mechanism stimulated by an increase venous return. This is supported by numerous studies that have shown increased filling pressure/volume accelerates the systemic heart of hagfish (Johansen 1960; Jensen 1961; Bloom et al. 1963; Chapman et al. 1963; Johnsson

and Axelsson 1996). And so, it may be more important to alter NA concentrations rather than AD concentrations during volume depletion as also appears to be the case during hypoxia/anaesthesia and surgery.

Volume depletion had marked acute cardiovascular effects as can be seen from the cardiovascular parameters measured during this manipulation (figure 2.4 Chapter Two). Despite the adrenergic response to volume depletion, hagfish were unable to maintain cardiovascular homeostasis as seen by immediate and sustained decreases in DA, PCV and pulse pressure (figure 2.4, Chapter 2). That SIV pressure decreased initially (although not significantly) and then recovered suggests some compensation does occur possibly through vasoactive means, as SIVs were shown in *in vitro* myography to often give the most potent responses to adrenergic stimulation.

There was no increase in HR at the corresponding time point to the initial AD surge on depletion, which is in agreement with the findings of *in situ* studies that show the systemic heart to be relatively insensitive to adrenergic stimulation.

That hagfish were not able to compensate for volume depletion was interesting in view of their large blood volume. In terms of absolute concentrations, plasma levels of NA were much lower during volume depletion that the levels measured during anaesthesia and surgery and may not have reached levels high enough to effect the appropriate cardiovascular changes required to buffer the effects of volume depletion. The hagfish cardiovascular system has been characterized as a low pressure, moderate flow system (Forster et al. 1991). Perhaps the acute ∼10% decrease in volume was not enough to elicit a significant induction of compensatory systems, although this seems unlikely in view of the compensation in cardiovascular function elicited during hypoxia (Forster et al. 1992). So although *E. cirrhatus* has the ability to boost its CO in response to hypoxia, it does not appear to be able to do the same during volume depletion.

It is possible that *E. cirrhatus* may have been unable to substantially increase plasma NA concentrations as a response to hypotension as stores of NA may have been depleted and vessels could have become desensitized due to the large releases of NA during surgery and anaesthesia. It is not known how fast hagfish can replenish catecholamines as no studies on catecholamine depletion or biosynthesis have been performed. It has been shown that the acute adrenergic response in fish can be substantially modified by prior history with respect to the extent of previous adrenergic responses (Reid et al. 1998). Despite the apparently suppressed adrenergic response to volume depletion, it is likely the response went some way to support cardiovascular function, it seems that function could have been further compromised without the observed increases in NA in view of the changes in NA concentration during volume depletion.

That cardiovascular depression persisted for a full 150 minutes after the induction of volume depletion suggests *E. cirrhatus* do not have other effective short term compensatory systems. Trout are able to nearly instantly mobilize a substantial volume of blood (20% of total blood volume) during hemorrhage by passive recoil of the microcirculation (Olson et al. 2003). Hagfish with their capacious sinus system that has a low rate of ex-

change with the central circulation and with their low pressure circulation are unlikely to have much reserve in their stressed volume and may not be naturally suited to compensate for depletion.

Plasma Catecholamine Concentrations During Volume Loading

Plasma catecholamine concentrations also displayed significant changes during volume loading experiments. Plasma NA levels decreased immediately upon induction of volume loading and remained suppressed for the duration of the experiment. This opposing adrenergic response to that observed during volume depletion further supports the hypothesis that NA exerts a pressor effect on the cardiovascular system of *E. cirrhatus*. That hagfish were able to regain cardiovascular homeostasis during volume loading (figure 2.4 Chapter 2) suggests that a reduction in basal NA concentration is an adaptive response to hypertension and may indicate a tonic control of the vasculature. Tonic control of vascular capacitance has been demonstrated in teleosts where it is primarily mediated by the sympathetic nervous system (Zhang et al. 1998). Wood and Shelton .(1980) found α-adrenergic blockade *in vivo* in rainbow trout revealed a considerable endogenous vasomotor tone resulting from latent adrenergic effects on systemic resistance.

As hagfish lack the advanced sympathetic nervous system of teleosts, it is likely they also lack this mechanism for controlling the capacitance of the vasculature. It is possible that basal circulating levels of catecholamines in hagfish could perform a function similar to that performed by the sympathetic nervous system in teleosts and exert a tonic control on the vasculature.

The decrease in central venous pressure may seem paradoxical in that CO would almost certainly be reduced, but this would provide an adaptive mechanism to help reduce blood pressure in the arterial side of the circulation.

The rapid decrease in central venous pressure and the concomitant decrease in NA concentrations during volume loading are in agreement with the hypothesis that a basal adrenergic tonus exists in the central venous vasculature.

How hagfish mediate the decreases in NA levels observed during volume depletion is unknown. It is possible they are able to reduce secretion of stored NA by some modulatory mechanism, such as the effect that adenosine exerts on AD release (Bernier et al. 1996), or they may be even able to regulate biosynthesis of NA. It is clear that this modulation of NA would benefit from further investigation.

The response of AD during volume loading was quite different to that of NA and was in contrast to the AD response seen during volume depletion as there were substantial increases in plasma concentrations of this substance. Although significant increases were not detected at early time points (possibly due to high pre-change levels) significant increases did occur at later time points with mean plasma AD concentrations in excess of 400nM.L^{-1} again in excess of previously reported plasma AD concentrations in hagfish.

That significantly increased DA and SIV pressures began to decline at the same time

points where AD concentrations were markedly elevated suggests an adaptive role for this substance in volume loading. *In vitro* myography work revealed that AD can elicit both constriction and dilatation of the central venous vasculature. If AD was to mediate depressor effects on the vasculature it could decrease the stressed blood volume and minimise the effects of volume loading on cardiovascular function. The response of AD to volume loading is also consistent with a cardio-protective role for this hormone as DA pressure was decreased with the observed increase in AD. This said, there are conflicting reports as to the actions of AD in hagfish. Although injections of AD in *in vivo* studies reduced systemic resistance in *M. glutinosa* (Axelsson et al. 1990), and branchial and systemic resistance in *E. cirrhatus* (Forster et al. 1992), application of AD to perfused hagfish gills elicited vasoconstriction (Axelsson et al. 1990; Sundin et al. 1994). The influence of AD on the vasculature hagfish is complex and further work is required to elucidate the intricacy of its actions.

AD secretion was obviously not compromised by the effects of surgery and anaesthesia one day previously, and there was a bias in terms of absolute concentration towards AD secretion with volume loading. Some authors have suggested that the bias towards NA release evoked by certain non cholinergic pituitary factors may reflect the storage levels of the two catecholamines (Bernier and Perry 1998). This does not explain the bias towards AD secretion seen during volume loading experiments which suggests a finer mechanism of control is operating than one simply based on storage levels. It has been suggested that increases in pressure may stimulate the release of catecholamines from the hearts of hagfish (Axelsson et al. 1990). However Perry et al (1993) found the chromaffin tissue of the PCV and systemic heart of *M. glutinosa* to be insensitive to changes in perfusion pressure in an *in situ* isolated heart preparation. That there were significant increases in AD during volume loading, but concomitant decreases in central venous pressure is in agreement with the findings of Perry et al (1993) and suggests a mechanism other than the direct effects of pressure on the chromaffin tissue is controlling secretion of AD.

Both NA and AD increase the filtration rate of the isolated perfused hagfish glomerulus via increase in pressure in the afferent glomerular vessels and so may exert a beneficial diuretic effect during volume loading (Fels et al. 1993). It is unclear how important the influence of catecholamines on glomerular filtration rate is in this model of volume loading as DA pressures initially increase with loading, although the increase is not significant. It is possible that increases in AD could mediate increased urine production as DA pressures decline after the transient increase, and sustained catecholamine levels could mediate a longer term diuresis in the event of prolonged volume loading.

Axelsson et al (1990) reported a concurrent increase in pooling of blood in the subcutaneous sinus and a decrease in the CO and blood pressure of hagfish that were apparently dying. This suggests that hagfish have an active mechanism that controls the return of blood to the central circulation from the sinus. It has been suggested that in situations where blood pressure is increased, blood may be forced into the sinus system (Forster 1997) and this is consistent with the observation by Satchell (1984) that the caudal heart

increased its frequency after exercise which may provide a mechanism for the return of blood to the central circulation. It is possible that during volume loading the mechanisms that regulate blood entry into or out of the sinus may relax which could contribute to the observed decrease in central venous pressure. Whether such a mechanism is passive or would be influenced by catecholamines is unknown. As there is a long turn over period between the blood contained in the subcutaneous sinus and the blood in the central circulation (Forster et al. 1989) combined with the lack of compensation in cardiovascular parameters during volume depletion it seems unlikely that hagfish can efficiently modulate shifts in blood volume out of the sinus and into the central circulation.

Although there are a large number of control systems that could potentially play a role in the redistribution of blood volume, few are likely to respond as rapidly as the adrenergic control system.

4.4.4 Effects of Exogenous NA on *In Vivo* Pressures

The injection of exogenous NA into the central venous compartment of *E. cirrhatus* produced significant increases in central venous pressure. There is also an indication that this treatment increases DA pressure possibly through an increase in CO, although data describing DA pressure was only collected from two hagfish.

As this was preliminary work, doses of NA were not calculated based on the animals blood volume. These experiments were performed simply to assess if NA would affect central venous pressures based on the changes in plasma catecholamines that had been observed in volume manipulation experiments and during surgery and anaesthesia. The findings from this experiment suggest NA does indeed exert a pressor effect on the central venous vasculature *in vivo* and is in agreement with the findings of the *in vitro* myography experiments. This supports the hypothesis that this NA would be recruited to help maintain or boost cardiovascular function when required by cardiovascular challenges such as hypoxia and volume depletion. It also supports the conclusion that release of NA would be maladaptive during volume loading.

There is an emphasis in the literature on the *in vivo* effects of AD on the cardiovascular parameters in hagfish, probably brought about by the established importance of humoral AD in teleost responses to cardiovascular challenge (Axelsson et al. 1990; Forster et al. 1992; Johnsson et al. 1996). As hagfish appear to lack the sympathetic innervation present in teleosts, the importance of NA as humoral effector of the responses that may normally be carried out by the sympathetic nervous system in higher vertebrates, appears to have been somewhat over looked and would benefit from further investigation

Appendix A

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Appendix B

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